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PRINCIPAL INVESTIGATOR: Laurel T. Holth, Ph.D.

CONTRACTING ORGANIZATION: University of Manitoba
Winnipeg, Manitoba, Canada R3E-OW3

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Laurel T. Holth, Ph.D.

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University of Manitoba
Winnipeg, Manitoba, Canada R3E-OW3

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The goal of this research is to study association of the estrogen receptor α (ER) with the nuclear matrix, and to identify nuclear matrix sites for ER. We constructed an ER fusion protein (GFP-ER) with a His6 tag, HA tag and the green fluorescent protein. Using fluorescent imaging techniques we studied the cellular localization of GFP-ER in the presence of various ER ligands in human breast cancer cell lines transiently transfected to express the GFP-ER protein. Striking differences in the patterns of distribution were observed. Isolation of GFP-ER associated proteins requires a large number of cell expressing GFP-ER. Therefore, a MCF7 stable cell line expressing GFP-ER under the control of an inducible promoter was constructed. Levels of GFP-ER expression can be controlled in this cell line, and this cell line has been carefully analyzed to determine the effect of GFP-ER expression on cell growth and gene expression. We demonstrated that GFP-ER association with the nuclear matrix increases with the length of exposure to estrogen, and that ER associated to the nuclear matrix is also binding to DNA, suggesting that nuclear matrix bound ER is transcriptionally active.

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FOREWORD

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INTRODUCTION

The subject of this research is the role of the nuclear matrix in estrogen receptorα (ER) regulated gene expression. ER is a nuclear matrix bound transcription factor that binds to nuclear matrix proteins (acceptors). The purpose of this research is to determine the role of the association of the nuclear matrix with the ER, specifically what proteins are involved and how ligand affects these interactions. There is evidence that the ER acceptors differ in antiestrogen-sensitive and -resistant breast cancer cells. Identification of these proteins may be informative in revealing whether an ER+ breast cancer is sensitive to antiestrogen therapy. We have designed a novel strategy to isolate and characterize the nuclear matrix acceptors for ER. Potentially this research will provide novel insights into the molecular mechanisms of gene regulation in general and, more specifically the mechanism of estrogen receptor action. This will potentially provide novel prognostic and diagnostic markers for breast cancer, and contribute to the development of new therapeutic applications.

BODY

A. Training accomplishments

This postdoctoral training grant has increased my research abilities and better prepared me to run my own laboratory. My research abilities have improved in that I believe I now ask more insightful questions and design better experiments. I am very aware of trying to design systems so that results are relevant to the biological/medical situation. I have gained increased awareness of issues relating to breast cancer, the U.S. Army conference was particularly useful in this.

As a result of this grant, I began a collaboration with Gordon Hager's lab at the NIH in Bethesda, including spending three weeks working in his lab. This collaboration made me aware of both the benefits and pitfalls of collaborating. It further enabled me to increase my circle of research contacts.

Many skills other than research ability are needed to become an independent researcher. Grant writing is an important skill that I worked on by applying for this award. Furthermore while holding this award I was able to train and supervise my own people. The first was a student from Germany, Uli Keller. The other was my technician Helen Zhao, who I also got to hire. In addition, having this award meant that I managed my own budget and supplies.

Finally this award provided me with the background and experience to be awarded the Clinical Chemistry Residency Position at the University of Manitoba. The research background provided by this U.S. Army award, in combination with the clinical background that I am currently obtaining through my residency in Clinical Chemistry will give me a unique combination of basic research and clinical skills. This combination will assist me in the future in the translation of basic research to the clinical setting for diagnostic and prognostic purposes.

B. Research Accomplishment

Localization of the Estrogen Receptor Fusion Protein

In the previous annual reports we reported that we had constructed a vector pCI-nGFP-ER, that when transiently transfected in human breast cancer cell lines directs the expression of an ER fusion protein we call GFP-ER. This ER fusion protein (GFP-ER) has three N-terminal tags that simplify its detection and isolation. The first, is the His_6 tag which will facilitate the isolation of the fusion protein using metal chelating columns or beads. Second, is an HA epitope tag which can be detected on western blots with anti-HA antibodies. Third, is the Green Fluorescent Protein (GFP) which makes it possible to observe GFP-ER in living cells using fluorescent and confocal microscopy

GFP-ER is a functional protein, as determined by transient transfection assays performed in the ER negative MDA MB 435A breast cancer cell line using the ERE-tk-CAT reporter vector.

Experiments examining the ligand dependent localization of GFP-ER were done in two ER+ cell lines MCF7 and T47D and two ER- cell lines, MDA MB 231 and MDA MB 435A. These human breast cancer cell lines were cultured either in the presence or absence of 17β-estradiol, 4-hydroxytamoxifen, or ICI 182 780. Transiently expressed GFP-ER protein was found only in the cells nucleus. In the transiently transfected cells cultured in phenol red free media with 5% twice charcoal stripped fetal bovine serum (i.e. no added ligand), a diffuse pattern of nuclear localization was seen. When 17β-estradiol was added to the media, the GFP-ER pattern became more punctate or speckled. The same effects were observed when 4-hydroxytamoxifen and ICI 182 780 were added to the media. In addition, ICI 182 780 altered the nucleocytoplasmic compartmentalization of the receptor, and caused partial accumulation in the cytoplasm in a process requiring continued protein synthesis. While these trends were observed in all four cell lines, variations were seen between cell lines. Clusters of GFP-ER were observed in the nucleoli of ER- cell lines, but not ER+ cell lines. The results of these experiments are detailed in a paper entitled "Direct Visualization of the Human Estrogen Receptor α Reveals a Role for Ligand in the Nuclear Distribution of the Receptor", published in Mol. Biol. of the Cell 10: 471-486.

Stable Cell Lines Expressing GFP-ER

In order to isolate the nuclear matrix proteins associated with GFP-ER it was necessary to prepare stable cell lines expressing this protein. We chose to regulate the expression of GFP-ER by introducing the tetracycline-on system into the desired cell lines. To use this system cells lines must first be stably transfected with the plasmid pUHD172-1neo that directs the expression reverse tetracycline-controlled transactivator (rtTA, a fusion between reverse Tetracycline repressor and activating domain of VP16 protein) and neomycin resistance gene. This was followed stably transfecting cells with GFP-ER cloned into the pUHD10-3 plasmid, placing GFP-ER under the control of a tetracycline inducible operon. In the absence of doxycycline, rtTA does not

activate the expression of GFP-ER, but when doxycycline is added, there is doxycycline concentration dependent activation of GFP-ER expression. We selected several stable MCF7(rTet) GFP-ER clones.

Characterization of the Stabily Transfected MCF7 Cell Line which inducibly expresses GFP-ER

As we are interested in isolating nuclear matrix proteins associated with GFP-ER as a method of identifying the nuclear matrix proteins normally associated with the endogenous ER , it is important that we do not over express GFP-ER to such an extent that it is causing abnormal cellular events, as our results would then be prone to artifact. It is therefore very important to characterize the expression of GFP-ER in the stabily transfected MCF7 cell line which inducibly expresses GFP-ER in the presence of doxycycline.

Western blot analysis of the induction of GFP-ER expression at 1 ug/ml DOX for various times after the addition of doxycycline was done to determine how long it took for expression levels to reach steady state. Seven hours after DOX is added GFP-ER expression is barely detectable, expression continues to increase up to the 48 hour time point, after which it seems to have reached equilibrium levels. This result suggests that experiments to isolate the nuclear matrix proteins associated with GFP-ER should be done at the least three days after the addition of doxycycline to allow cells to go through one cell cycle after GFP-ER expression has reached equilibrium levels. The reasoning behind allowing at least one round of the cell cycle after reaching equilibrium values is that many DNA binding proteins only have access to DNA during S phase, and we want the GFP-ER to establish the same associations as the endogenous ER.

Western blot analysis of the doxycycline dose response of GFP-ER expression showed the following. No expression of GFP-ER is detected in the absence of doxycycline. An increase in GFP-ER expression is observed with increasing doxycycline concentrations, with this effect plateauing at 1 ug of doxycycline per ml of media.

We further looked at the GFP-ER expression using fluorescent activated flow cytometry or FACS, taking advantage of the fluorescence of GFP. In the absence of estrodiol, doses of 0.1, 0.5 and 1.0ug/ml of DOX induced GFP-ER expression in 31, 58 and 68.5% of clone 11 cells respectively. We exposed cells treated as above to 10nM of E2 for 24h. This resulted in levels of GFP-ER expression being decreased about 32%. As ER has been shown to be degraded more rapidly in the presence of estrogen (Nawaz et al., PNAS 96: 1858-1862), this result indicates that the GFP tag is not interfering with estrogen induced degradation of ER. Using FACS to look at cell cycling and GFP-ER expression simultaneously, showed that after exposure to 10nM E2 for 24h, the GFP-ER level was decreased in both G0+G1 and S+G2+M phases cell compared with the no E2 group.

FACS analysis was also used to determine if doxycycline, and the induction of GFP-ER expression effected cell cycling. The experiments were done using the parent MCF7 cell line (clone 89) which has not been transfected

with GFP-ER (but have been transfected with pUHD172-1neo, so are expressing rtTA) and the MCF7 cell line stabily transfected with the doxycycline inducible GFP-ER gene (clone 11). In both cell lines doxycycline had no effect on cell cycling. Exposure to 10 nM estradiol for 3 days increased the percentage of cells in S+G2+M, 1.5 - 2 fold in both cell lines at doxycycline concentrations ranging from 0 to 1.0 ug/ml. These results indicate that GFP-ER expression is not interfering with cell cycle at these levels of expression. Likewise FACS analysis was used to check cell growth under the same conditions. Doxycycline did not effect cell growth in either clone 89 or clone 11 cells, while 10 nM estradiol for 24 hrs decreased doubling times 1.5 - 2.7 fold in both cell lines as would be expected.

In order to determine if estrogen induction of endogenous genes was perturbed by the expression of GFP-ER, the induction of endogenous genes with estrogen was examined using Northern blots prepared with RNA from time course experiments and probed with the cDNA for *c-myc* and *pS2*. The experiments were done using the parent MCF7 cell line (clone 89), and the MCF7 cell line stabily transfected with the doxycycline inducible GFP-ER gene (clone 11). 10nM of E2 increased *c-myc* mRNA level in both clone 89 and clone 11 cell lines. Adding 0.1, 0.5ug/ml of DOX to clone 11 cells to induce GFP-ER expression did not affect the induction pattern of *c-myc* mRNA. *c-myc* mRNA reached highest level in the cell lines when the cells were exposed to 10nM of E2 for 1h and then gradually dropped down to the original level after 24h exposure to E2.

In both clone 89 and clone 11 cell lines, *pS2* mRNA was increased by 10nM E2. The longer the time exposed to E2, the higher the level of *pS2* mRNA, with the longest time point being 24 hours. The *pS2* mRNA levels in both cell lines increased 2-4 times over the levels expressed in the absence of E2. Addition of 0.1-1.0ug/ml of DOX to clone 11 did not affect the E2 induction of *pS2* mRNA.

Estrogen Increases Association of GFP-ER with Nuclear Matrix

Transiently expressed GFP-ER associates with the nuclear matrix. As we are planning to use our stable cell lines to isolate the nuclear matrix acceptor for the ER, using the GFP-ER protein, it was important to verify that GFP-ER was also associating with the nuclear matrix in the stabily transfected MCF7 cell line. We were further interested in the effect of estrogen on GFP-ER association with the nuclear matrix. A western blot of nuclear matrix proteins isolated at various times after the addition of estrogen was prepared. The blot was probed with the HA antibody which identifies the N-terminus of GFP-ER. In the absence of estrogen some GFP-ER was found associated with the nuclear matrix. As the length of exposure to estrogen was increased so did the amount of GFP-ER found in the nuclear matrix. Thus estrogen very clearly affects the association of GFP-ER with the nuclear matrix. We expect to find that at least some of the nuclear proteins associated with the ER will change depending on the ligand associated with the ER.

Purification of GFP-ER.

The main goal of this project is to find the nuclear matrix acceptor(s) for the ER. This will be done by isolating nuclear matrix and then crosslinking it, followed by isolation of GFP-ER and its associated crosslinked proteins. Therefore, of primary importance to this project is the isolation and purification of GFP-ER.

In preliminary studies reported in our 1997 annual report, we showed isolation of GFP-ER from MCF-7 cells transiently transfected with GFP-ER expression plasmid, was possible with the Talon metal affinity resin. Metal affinity resins can be used to isolate proteins with His_6 tags. Further purification of GFP-ER was planned by additionally doing immunoprecipitation with anti-HA antibodies and protein A.

As the initial results on purification of GFP-ER from the transiently transfected cells were very positive, we hoped that the purification from the stable cell lines expressing GFP-ER would be fairly straight forward. Unfortunately the Talon metal affinity resin purification was much less efficient on the stable cells expressing GFP-ER. One plausible reason for this was the isolation of GFP-ER from the transiently transfected cells was done 24-48 hours after transfection with the transients while with the stable lines GFP-ER had been expressing for at least three days, and that this difference in time may have resulted in it becoming more tightly associated with other components of the cell. A second reason for the differences is that the expression levels on an individual cell basis are lower in the stable cell lines than in the transients, the GFP-ER over expression in the transients may have resulted in some GFP-ER that was only loosely associated with cellular structures and therefore more soluble. We will be trouble shooting and optimizing the isolation of GFP-ER by altering buffer conditions for the extractions and trying metal affinity resins from other sources.

ER binds DNA while associated with the Nuclear Matrix

We have published (Samuel *et al.,* Cancer Res. 58: 3004-3008) that the endogenous nuclear matrix bound ER of MCF-7 cells was crosslinked to DNA with cisplatin (an antitumor drug). This paper provides evidence that the nuclear matrix is not merely a storage site for inactive transcription factors, but that the nuclear matrix associated ER is functional in the sense that it is bound to DNA.

ACKNOWLEDGMENTS

I would like to acknowledge the following individuals for their contributions to this work, the head of my lab and my mentor, Dr. James Davie, our collaborators, Dr. Gordon Hager and Dr. Han Htun (N.I.H.). Thanks also to Dr. Leigh Murphy (Univ. of Manitoba) for helpful discussions and to Helen Zhao (technician), Uli Keller (student), and Laura Hart (student) for their efforts on this project.

APPENDICES

I. KEY RESEARCH ACCOMPLISHMENTS

- Developed a fusion protein (GFP-ER) between the human estrogen receptor α (ER) and green fluorescent protein (GFP).

- Demonstrated that GFP-ER is functional as a ligand dependent transcription factor.

- Demonstrated that GFP-ER associates with the nuclear matrix, and that this association increases with exposure to estrogen.

- Analyzed the cellular localization of GFP-ER in four human breast epithelial cell lines, two ER+ lines and two ER- lines. Found:
    - In all cell lines GFP-ER is found only in the nucleus in the absence of ligand.
    - The presence of either agonist or antagonist ligand results in the nuclear distribution of GFP-ER becoming punctate.
    - The presence of the antagonist ligand ICI 182 780 alters nucleocytoplasmic compartmentalization of the receptor, and causes partial accumulation in the cytoplasm in a process requiring continued protein synthesis.
    - GFP-ER was observed in the nucleolar region of ER- cell lines, but not ER+ cell lines,

- Developed a stable line of MCF7 cells that expressed GFP-ER under the control of a Tet inducible promoter system. Demonstrated that expression of GFP-ER in these lines did not perturb normal estrogen regulated gene expression or cell growth. Therefore this is an ideal system to now use to study ER *in situ*.

- ER binds DNA while associated with the nuclear matrix. This provides evidence that the nuclear matrix is not merely a storage site for inactive transcription factors, but that the nuclear matrix associated ER is functional in the sense that it is bound to DNA.

II. REPORTABLE OUTCOMES

1. In the last three years, we have published the following manuscripts:

Davie, J.R., S.K. Samuel, V.A. Spencer, L.T. Holth, D.N. Chadee, C.P. Peltier, J.-M. Sun, H.Y. Chen, and J.A. Wright. (1999). Organization of chromatin in cancer cells: role of signalling pathways. Biochem. Cell Biol. 77:1-11.

Htun, H.*, L.T. Holth* **(*contributed equally to this work)**, D. Walker, J.R. Davie and G.L. Hager. (1999). Direct Visualization of the Human Estrogen Receptor α Reveals a Role for Ligand in the Nuclear Distribution of the Receptor. Mol. Biol. of the Cell 10: 471-486.

Holth, L.T., D.N. Chadee, V.A. Spencer, S.K. Samuel, J.R. Safneck and J.R. Davie. (1998). Chromatin, nuclear matrix and the cytoskeleton: Role of Cell structure in neoplastic transformation. International Journal of Oncology 13:827-837.

Samuel, S.K., V.A. Spencer, L. Bajno, J.-M. Sun, L.T. Holth, S. Oesterreich and J.R. Davie. (1998). *In Situ* Cross-Linking by Cisplatin of Nuclear Matrix-bound Transcription Factors to Nuclear DNA of Human Breast Cancer Cells. Cancer Research 58: 3004-3008.

Walia, H., H.Y. Chen, J.-M. Sun, L.T. Holth and J.R. Davie. (1998). Histone acetylation is required to maintain the unfolded nucleosome structure associated with transcribing DNA. Journal of Biological Chemistry 273:14516-14522.

Davie, J.R., S. Samuel, V. Spencer, L. Bajno, J.-M. Sun, H.Y. Chen, and L.T. Holth. (1998). Nuclear matrix: application to diagnosis of cancer and role in transcription and modulation of chromatin structure. Gene Therapy and Molecular Biology 1:509-528.

Holth, L.T., J.-M. Sun, A.S. Coutts, L.C. Murphy and J.R. Davie. (1997). Estrogen Receptor Diminishes DNA-Binding Activities of Chicken GATA-1 and CACCC-Binding Proteins. DNA and Cell Biology 16:1477-1482.

2. In the past three years, we have published the following Abstracts:

Davie, J.R., S. Samuel, V. Spencer, L. Bajno, J.-M. Sun, H. Chen, **L.T. Holth**. Keystone Meeting (1998). "Transcriptional Modulators and the Nuclear Matrix".

Holth, L.T., H. Htun, G.L. Hager, and J.R. Davie. Era of Hope - U.S. Army Breast Cancer Research Meeting. Washington, D.C. (1997). "Interaction of the Estrogen Receptor with the Nuclear Matrix in Breast Cancer".

Holth, L.T., A.E. Thorlacius and R. Reeves. Era of Hope - U.S. Army Breast Cancer Research Meeting. Washington, D.C. (1997). "Effects of Epidermal Growth Factor and Estrogen on the Regulation of the HMG-I/Y Gene in Human Mammary Epithelial Cell Lines".

Sullivan, T.M., A.V. Lee, S.A.W. Fuqua, **L. Holth**, S.K. Samuel, J.R. Davie, and S. Oesterreich. 20th Annual San Antonio Breast Cancer Symposium (1997). "HET is a nuclear matrix protein involved in breast cancer growth regulation".

Htun H., D. Walker, R. Wolford, J. Barsony, **L. Holth**, J.R. Davie, and G.L. Hager. Cold Spring Harbor Meeting on Transcription (1997). "Ligand-dependent targeting of steroid receptors to nuclear structures studied in cultured living cells".

3. This award resulted in the development of the GFP-ER fusion protein, in which the human estrogen receptor is fused to the green fluorescent protein. This protein also contains HA and His6 tags.

4. This award resulted in the development of an MCF7 cell line which is stably transfected with the gene for the expression of the GFP-ER fusion protein under the control of a tetracycline inducible promoter.

5. The work supported by this award was the bases of a successful grant application to the Medical Research Council of Canada / Manitoba Health Research Council.

6. I was awarded a residency in Clinical Chemistry, based in part on the experience I gained from this U.S. Army Fellowship.

7. I was able to provide a technician with a job for one year because of this award. The research done during this award was the bases for the grant listed in point 5, and therefore this technician's continued employment.

8. This award provided me with supervisory experience, as it involved me in the training and supervision of my technician and several students.

REVIEW / SYNTHÈSE

Organization of chromatin in cancer cells: role of signalling pathways

J.R. Davie, S.K. Samuel, V.A. Spencer, L.T. Holth, D.N. Chadee, C.P. Peltier, J.-M. Sun, H.Y. Chen, and J.A. Wright

Abstract: The role of mechanical and chemical signalling pathways in the organization and function of chromatin is the subject of this review. The mechanical signalling pathway consists of the tissue matrix system that links together the three-dimensional skeletal networks, the extracellular matrix, cytoskeleton, and nuclear matrix. Intermediate filament proteins are associated with nuclear DNA, suggesting that intermediate filaments may have a role in the organization of chromatin. In human hormone-dependent breast cancer cells, the interaction between cytokeratins and chromatin is regulated by estrogens. Transcription factors, histone acetyltransferases, and histone deacetylases, which are associated with the nuclear matrix, are components of the mechanical signalling pathway. Recently, we reported that nuclear matrix-bound human and chicken histone deacetylase 1 is associated with nuclear DNA in situ, suggesting that histone deacetylase has a role in the organization of nuclear DNA. Chemical signalling pathways such as the Ras/mitogen-activated protein kinase (Ras/MAPK) pathway stimulate the activity of kinases that modify transcription factors, nonhistone chromosomal proteins, and histones. The levels of phosphorylated histones are increased in mouse fibroblasts transformed with oncogenes, the products of which stimulate the Ras/MAPK pathway. Histone phosphorylation may lead to decondensation of chromatin, resulting in aberrant gene expression.

Key words: histone acetylation, histone phosphorylation, nuclear matrix, cytoskeleton, histone deacetylase, cancer.

Résumé : Cette revue porte sur le rôle des voies de signalisation mécanique et chimiques dans l'organisation et la fonction de la chromatine. La voie de signalisation mécanique comprend le système de matrice tissulaire liant ensemble les réseaux squelettiques tridimensionnels : la matrice extracellulaire, le cytosquelette et la matrice nucléaire. Les protéines des filaments intermédiaires sont associées à l'ADN nucléaire, ce qui suggère que les filaments intermédiaires joueraient un rôle dans l'organisation de la chromatine. Dans les cellules de tumeurs du sein hormonodépendantes, les oestrogènes règlent l'interaction entre les cytokératines et la chromatine. Des facteurs de transcription, les histones acétyltransférases et les histones désacétylases, tous associés à la matrice nucléaire, sont des éléments de la voie de signalisation mécanique. Récemment, nous avons montré que l'histone désacétylase I liée à la matrice nucléaire chez l'humain et le poulet est associée à l'ADN nucléaire in situ, ce qui suggère que l'histone désacétylase joue un rôle dans l'organisation de l'ADN nucléaire. Les voies de signalisation chimiques, telle la voie de la Ras/MAPK (protéine kinase activée par Ras ou un mitogène), stimulent l'activité de kinases qui modifient des facteurs de transcription, des histones et des protéines chromosomiques non histones. Les taux d'histones phosphorylées sont augmentés dans des fibroblastes de souris transformés par des oncogènes dont les produits stimulent la voie de la Ras/MAPK. La phosphorylation des histones pourrait entraîner la décondensation de la chromatine, ce qui résulterait en une expression génique aberrante.

Mots clés : acétylation des histones, phosphorylation des histones, matrice nucléaire, cytosquelette, histone désacétylase, cancer.

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Abbreviations: ARBP, attachment region binding protein; cisplatin, *cis*-diamminedichloroplatinum; ER, estrogen receptor; IGCs, interchromatin granule clusters; MAP, mitogen-activated protein; MARs, matrix attachment regions; MeCP2, methyl-CpG-binding protein 2; NMBCs, nuclear matrix proteins in breast cancer; NuRD, nucleosome remodelling histone deacetylase complex; Ras/MAPK, Ras/mitogen-activated protein kinase.

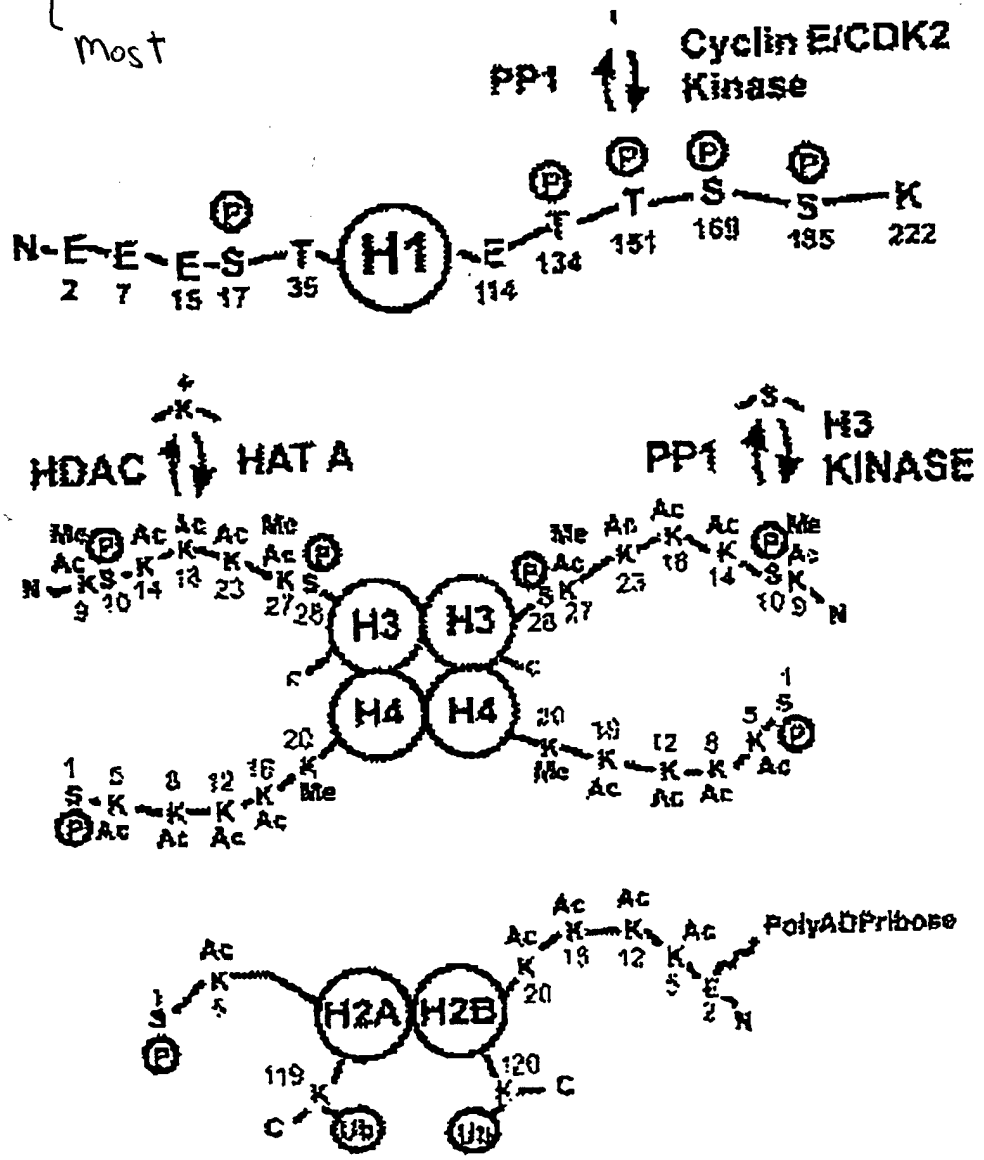
J.R. Davie,[1] S.K. Samuel, V.A. Spencer, L.T. Holth, D.N. Chadee, C.P. Peltier, J.-M. Sun, H.Y. Chen, and J.A. Wright. Manitoba Institute of Cell Biology and the Department of Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, MB R3E 0V9, Canada.

[1]Author to whom all correspondence should be addressed (e-mail: Davie@cc.umanitoba.ca).

methylation (Me)

Fig. 1. Sites of postsynthetic modifications on the histones. The structures of the H2A–H2B dimers, $(H3–H4)_2$ tetramers, H1, and the sites of modification are shown. The modifications shown are acetylation (Ac), phosphorylation (P), ubiquitination (Ub), and poly(ADPribosyl)ation. All of these modifications are reversible. The enzymes catalyzing reversible acetylation and phosphorylation are shown (HAT, histone acetyltransferase; HDAC, histone deacetylase; CDK2, cyclin-dependent protein kinase 2; PP1, protein phosphatase 1).

Most



Chromatin structure and histone modifications

The basic repeating unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped around a histone octamer (Luger et al. 1997). The histone octamer contains two each of the core histones H2A, H2B, H3, and H4. The core histones of the octamer are arranged as a $(H3–H4)_2$ tetramer and two H2A–H2B dimers positioned on both sides of the tetramer. The core histones have a similar structure with a basic N-terminal unstructured domain, a globular domain organized by the histone fold, and a C-terminal unstructured tail (Fig. 1). The N-terminal tails of the core histones, which emanate from the nucleosome at about every 20 bp, are not involved in maintaining the structural integrity of the nucleosome (Luger et al. 1997; Rhodes 1997). A fifth class of histone, the H1 histones, binds to the DNA joining nucleosomes together and to core histones. H1 has a tripartite structure consisting of a central globular core and lysine rich N- and C-terminal domains. The globular domain binds to one linker DNA strand as it exits or enters the nucleosome and to nucleosomal DNA near the dyad axis of symmetry of the nucleosome (Zhou et al. 1998). The H1 histones are a group of several subtypes that differ in amino acid sequence (Parseghian et al. 1994).

The H1 histones and the N-terminal domains of the core histones stabilize the higher order folding of the chromatin fiber (Van Holde and Zlatanova 1996; Hansen 1997). The

capacity of these histones to compact the chromatin fiber is reversibly controlled by multiple postsynthetic modifications. Amino acids located in the N- and C-terminal tails of the H1 histones are susceptible to phosphorylation and poly(ADP-ribosyl)ation. The N-terminal domains of the core histones are modified by acetylation, methylation, phosphorylation, and ADP-ribosylation, while the C-terminal domains of histones H2A and H2B can be ubiquitinated (Davie and Chadee 1998) (Fig. 1). Although there has been considerable interest in histone acetylation and the enzymes catalyzing this reversible process, it is important to note that a dynamically acetylated histone (e.g., H3) may also be phosphorylated and methylated (Davie 1998).

It has been proposed that the N-terminal tails undergo an induced folding when in contact with other proteins or DNA. Such inter- and intra-fiber interactions of the core histone tails would be involved in stabilizing the higher order folding of chromatin. Modification of the tails would interfere with folding of the N-terminal tail and interactions with proteins and (or) DNA, destabilizing higher order chromatin organization (Garcia-Ramirez et al. 1995; Hansen et al. 1998). Modification of the N-terminal tails of the core histones may also alter their interactions with architectural proteins (Tjian and Maniatis 1994). For example, acetylation of the N-terminal tails may disrupt interactions with nonhistone chromosomal proteins (Edmondson et al. 1996; Palaparti et al. 1997; Trieschmann et al. 1998; Parkhurst 1998; Nightingale et al. 1998).

The chromatin fiber is organized into loops where the DNA (the matrix attachment region (MAR)) at the base of the loop is bound to nuclear matrix proteins (Davie 1995; Samuel et al. 1998; de Belle et al. 1998) (Fig. 2). A chromatin loop may contain one or several genes. A comparison of the DNA sequences of MARs shows that they do not share extensive sequence homology; however, MAR–DNA sequences have high bending potential and may act as topological sinks (Bode et al. 1996; Benham et al. 1997).

Transcriptionally active chromatin

Chromatin loops containing expressed genes have a decondensed configuration that is sensitive to DNase I digestion, while loops with repressed genes have a condensed structure (Fig. 2). Extended chromatin loops with decondensed (30-nm fiber) regions, which are presumably transcribed, have been observed in G1 phase nuclei of Chinese Hamster Ovary cells (Belmont and Bruce 1994). Interestingly, these decondensed chromatin regions were often found near interchromatin granule clusters (IGCs), ribonucleoprotein structures that serve as storage sites for splicing and transcription factors (Hendzel et al. 1998; Misteli and Spector 1998). Highly transcribed genes are located near IGCs (Misteli and Spector 1998), and highly, dynamically acetylated histones are associated with the chromatin positioned near these structures (Hendzel et al. 1998).

Transcribing chromatin is selectively bound to the nuclear matrix (Davie 1997; Stein et al. 1997). Multiple dynamic MARs attach transcribed chromatin regions to the nuclear matrix (see inset in Fig. 2); these MARs are different from those found at the base of loops (Davie 1997). Nuclear matrix proteins, including nuclear matrix-bound transcription factors (e.g., YY1 and AML), the transcription machinery, and histone-modifying enzymes (e.g., histone acetyltransferases and deacetylases) are thought to mediate the dynamic attachments between transcriptionally active chromatin and the nuclear matrix (Davie 1997; Jackson 1997; Stein et al. 1997; McNeil et al. 1998).

Signalling pathways and the organization and function of chromatin

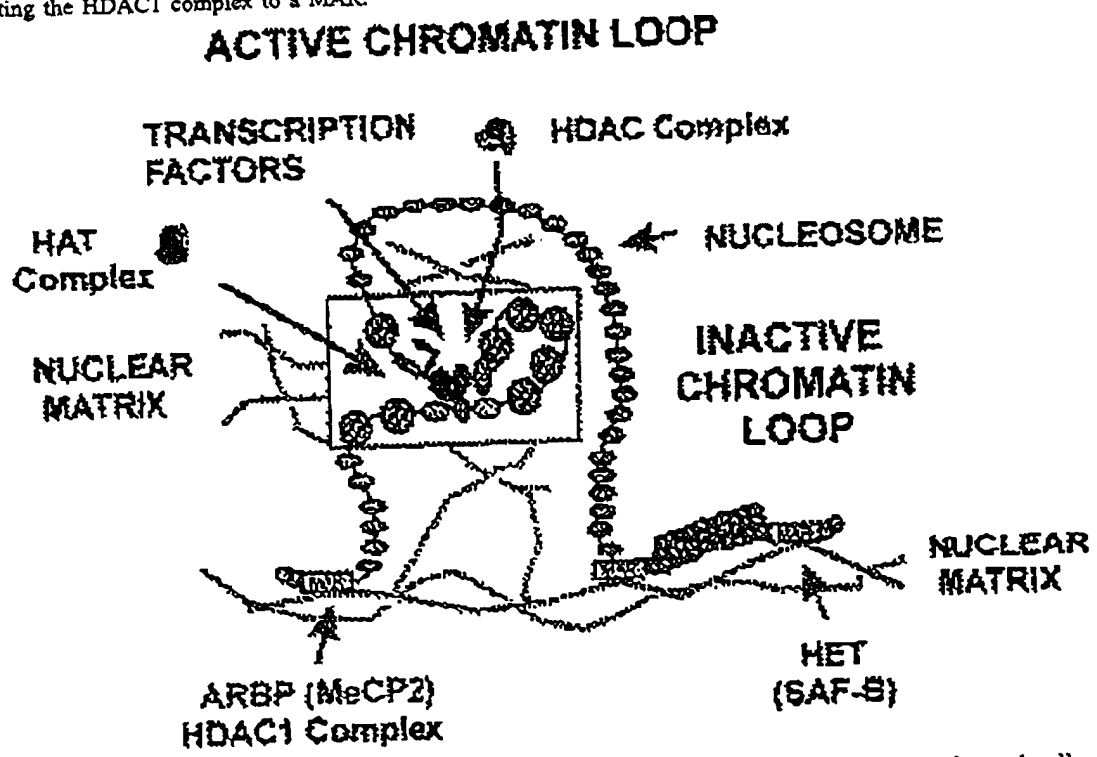
Both mechanical and chemical signalling pathways are involved in transmitting information from the cell's exterior to chromatin. The mechanical signalling pathway consists of the tissue matrix system that links together the three-dimensional skeletal networks, the extracellular matrix, cytoskeleton and nuclear matrix (Penman 1995; Maniotis et al. 1997). The dynamic tissue matrix system governs cell and nuclear shape. The mechanical signalling pathway has a role in controlling cell cycle progression and gene expression (Chen et al. 1997; Huang et al. 1998; Myers et al. 1998). Chemical signalling pathways, such as the Ras/mitogen-activated protein kinase (Ras/MAPK) pathway, can stimulate the activity of kinases that modify transcription factors, nonhistone chromosomal proteins, and histones (Mahadevan et al. 1991; Chadee et al. 1995; Wasylyk et al. 1998). Activation of the Ras/MAPK pathway results in the alteration of chromatin structure and gene expression. The tissue matrix and chemical signalling pathways are not independent and one signalling pathway can affect the other (Reszka et al. 1995; Yujiri et al. 1998; Aplin and Juliano 1999). We are interested in how these signalling pathways alter the organization and structure of chromatin of malignant cells.

Chromatin and nuclear structure of oncogene-transformed mouse fibroblasts

We have observed two features of nuclear organization in oncogene-transformed mouse fibroblasts. First, oncogene-transformed mouse fibroblasts with a high metastatic potential have an altered nuclear morphology (Fischer et al. 1998). Second, oncogene-transformed mouse fibroblasts have a relaxed chromatin structure (Laitinen et al. 1990; Chadee et al. 1995).

The extent of changes in nuclear morphology of oncogene-transformed mouse fibroblasts correlated with their metastatic potential (Fischer et al. 1998). It should be noted that pathologists have long used aberrant appearing nuclei as a diagnostic marker for cancer. Since the nuclear matrix has a role in nuclear shape, we were interested in how the profiles of nuclear matrix proteins changed with the metastatic potential of oncogene-transformed cells. Two sets of cell lines were used. The first set was derived from 10T½ fibroblasts (Egan et al. 1987*a*). These lines were transfected with H-*ras* and the transformed foci gave rise to the cell lines CIRAS-1, -2, -3. Using criteria such as experimental metastasis assays, tumour latency, anchorage-independent growth, and frequency of spontaneous metastasis (Samuel et al. 1992, 1993), it was determined that CIRAS-1 was poorly

Fig. 2. Organization of nuclear DNA. MAR-DNA binds to nuclear matrix proteins, organizing chromatin into loop domains. The inset is meant to show the multiple dynamic interactions between the nuclear matrix and regulatory and transcribed DNA sequences. Histone acetyltransferase (HAT) and histone deacetylases (HDAC) multiprotein complexes are recruited to sites of transcription. TFs are transcription factors, some of which are associated with the nuclear matrix (e.g., ER, estrogen receptor). HET/SAF-B is a MAR-binding protein. ARBP/MeCP2 is also a MAR binding protein that is a repressor. The nuclear matrix-associated ARBP/MeCP2 is shown recruiting the HDAC1 complex to a MAR.



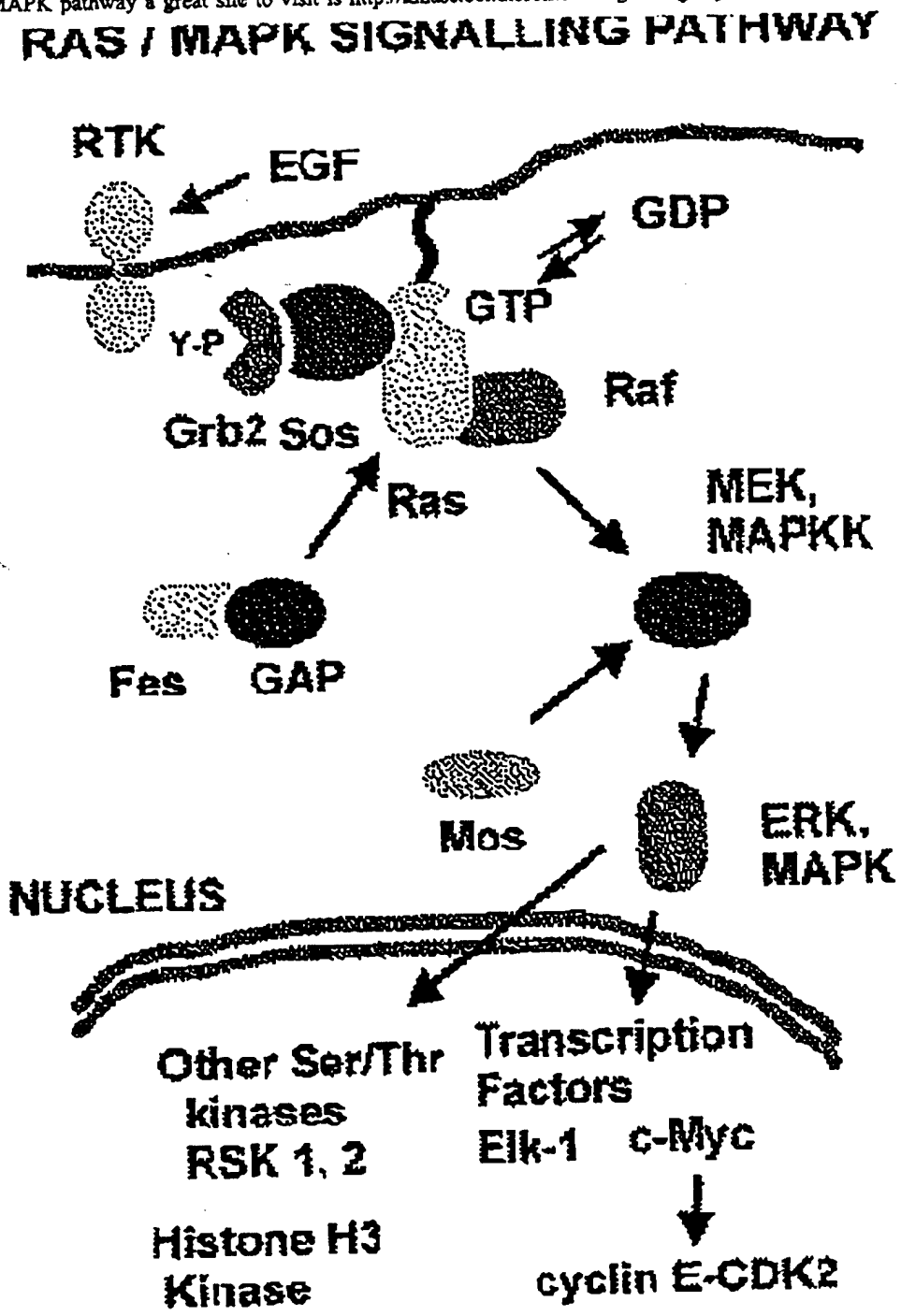
metastatic, CIRAS-2 had intermediate metastatic properties, and CIRAS-3 was highly metastatic. The second panel of cell lines was derived from NIH 3T3 fibroblasts transfected with oncogenes encoding kinases (serine/threonine or tyrosine) (Egan et al. 1987*b*). Criteria for selection were similar to that of the 10T½ derived fibroblast cell lines. From this, it was determined that 3T3/*raf* was poorly metastatic, while 3T3/*fes* was highly metastatic. Using these cell lines, we found that the highly metastatic cell lines (CIRAS-3, 3T3-*fes*) had similar nuclear matrix profiles that were different from the poorly metastatic cell lines (CIRAS-1, 3T3-*raf*) (Samuel et al. 1997*a*). Clearly, these data suggest that there is a unique nuclear matrix profile for each stage of malignancy regardless of transformation agent. Further, the major changes in nuclear matrix proteins patterns observed in oncogene-transformed cells with high metastatic potential correlated with altered nuclear morphology.

Ras- and *myc*-transformed mouse fibroblasts have less condensed chromatin than that of the parental cells. The relaxation of chromatin in these oncogene-transformed cells could result from alterations in the postsynthetic modification of histones. We found an increased level of phosphorylated H1, and more recently phosphorylated H3, in mouse fibroblasts transformed with oncogenes, the products of which stimulate the Ras/MAPK pathway, or constitutively active mitogen-activated protein (MAP) kinase (Chadee et al. 1995, 1999; Taylor et al. 1995). The extent of changes in the level of phosphorylated H1 or H3 histones was similar for all oncogene-transformed cells analyzed and appeared to be independent of the cell's metastatic potential, which was determined by experimental metastasis assays (Taylor et al. 1995; Chadee et al. 1999). It was hypothesized that the persistent activation of the MAP kinase pathway in these cells may have altered the cyclin E-associated H1 kinase activity resulting in the observed increase in phosphorylation of H1 (Chadee et al. 1995) (Fig. 3). Fibroblasts lacking the tumour suppressor Rb also exhibit an increased level of phosphorylated H1 and relaxed chromatin structure and deregulation of CDK2 may be directly involved (Herrera et al. 1996). Activation of the Ras/MAPK pathway also results in the activation of a currently unidentified histone H3 kinase, resulting in the phosphorylation of H3 (Mahadevan et al. 1991). H1 and H3 phosphorylation destabilizes higher order chromatin structure. Therefore, H1 and H3 phosphorylation may lead to decondensation of chromatin, resulting in access to transcription factors and (or) removal of blocks in elongation (Davie and Chadee 1998). The net result of the relaxation of chromatin structure could be aberrant gene expression.

We found that phosphorylation of histone H1b in mouse fibroblasts was dependent upon transcription and replication processes. Histone H1b phosphorylation is the only histone modification known to be dependent upon both of these nuclear processes (Chadee et al. 1997). This observation provides evidence that histone H1b is associated with transcribed chromatin. H1b is the mouse H1 subtype with the most highly

Please define (MAP Kinase Kinase)$_5$

Fig. 3. The Ras/MAPK signalling pathway. MAPKK and MAP kinase (MAPK) are also called MEK and ERK, respectivcly. For more information on the MAPK pathway a great site to visit is http://kinase.oci.utoronto.ca/signallingmap.html.



phosphorylated isoforms (Lennox et al. 1982). Increased phosphorylation of H1b in comparison to normal lung tissues has been observed in mouse Lewis lung carcinoma (Lennox et al. 1982). Based upon the results of Jerzmanowski and Cole (1992), we proposed that the transcription or replication processes were required to expose H1b to the cyclin E-CDK2 kinase (Chadee et al. 1997). Once phosphorylated, H1b would contribute to the relaxation of the transcribed chromatin fiber.

Mechanical signalling pathways and organization of nuclear DNA in human breast cancer cells

Intermediate filaments extend from the nucleus to the plasma membrane (Georgatos and Maison 1996) and are enmeshed with and penetrate the nuclear lamina (Ward et al. 1984; Nickerson et al. 1990; Penman 1995; Wang et al.

1996). Intermediate filaments are associated with nuclear DNA in vivo (Ward et al. 1984; Olinski et al. 1987), and in vitro studies provide evidence that intermediate filaments have DNA sequence preferences (Wang et al. 1996). It is conceivable that intermediate filaments communicate signals from the extracellular matrix to nuclear DNA, resulting in changes in chromatin organization and perhaps function. Studies by Maniotis et al. (1997) demonstrated that the intermediate filament network was sufficient to transmit mechanical stress to the nucleus.

It has been proposed that the filament ends of the intermediate filaments associate with DNA at the nuclear lamina (Ward et al. 1984). In support of this idea, transmission electron micrographs show intermediate filaments uniting with nuclear lamina (Capco et al. 1982). Further, immunolocalization studies show that intermediate filaments are located throughout the cytoplasm and surrounding nuclei of breast cancer cells (Sapino et al. 1986). It is interesting to note that one of the primary targets of cisplatin in HeLa cells is the inner side of the nuclear double membrane (Khan and Sadler 1978).

Recently, we presented evidence that the intermediate filament proteins cytokeratins K8, K18, and K19 are associated with nuclear DNA of human breast cancer cells (Spencer et al. 1998). These studies were done with the cross-linking agent *cis*-diamminedichloroplatinum (cisplatin), an agent that preferentially cross-links nuclear matrix proteins to MAR DNA in situ (Ferraro et al. 1992; Holth et al. 1998). We found that the levels of cytokeratins associated with nuclear DNA and the nuclear matrix were drastically reduced in an estrogen receptor (ER)-positive, hormone-dependent breast cancer cell line grown in acutely estrogen-depleted conditions, while treatment of these estrogen-starved cells with estrogen restored cytokeratin interactions with nuclear DNA (Coutts et al. 1996; Spencer et al. 1998). This study also showed that cells chronically depleted of estrogen developed a hormone-independent phenotype and had greater levels of cytokeratins K8, K18, and K19 associated with nuclear DNA and nuclear matrix compared to levels observed in the control cell line grown in the presence of estrogen (Coutts et al. 1996; Spencer et al. 1998). These studies show that the interactions between intermediate filaments and nuclear DNA are regulated by estrogens in hormone-dependent breast cancer cells, but this regulation is lost in hormone-independent, ER-positive breast cancer cells. In hormone-dependent cells, estrogen-regulated interactions between intermediate filaments and nuclear DNA could manipulate the organization of chromatin.

As discussed previously, the nuclear matrix has a role in determining nuclear morphology and organization of chromatin. Pathologists routinely use altered nuclear morphology as a marker for breast cancer. Further, several studies have shown that nuclear matrix proteins may serve as informative biomarkers in the diagnosis and prognosis of cancer (for review see Replogle-Schwab et al. 1996; Carpinito et al. 1996; Hughes and Cohen 1999). Therefore, it was of interest to us to identify nuclear matrix proteins that could be used as diagnostic and prognostic indicators for breast cancer. In a recent study in our laboratory, the two-dimensional gel patterns of nuclear matrix proteins of a variety of human breast cancer cell lines that were either ER-positive and hormone-dependent or ER-negative and hormone-independent (was) analyzed. MCF-10A1, a spontaneously immortalised human breast epithelial cell line was used as a control (Samuel et al. 1997*b*). Specific breast cancer nuclear matrix proteins exclusive to ER status were identified. We refer to these proteins as nuclear matrix proteins in breast cancer (NMBCs), using the nomenclature proposed by Pienta and colleagues (Khanuja et al. 1993). Five NMBCs exclusive to ER+ cell lines and one NMBC exclusive to the ER- cell lines were identified (Samuel et al. 1997*b*). As we are interested in the diagnostic and prognostic potential of these proteins in human breast cancer, we then looked at tumour tissue samples. Using ER+ and ER– human breast tumours, we were able to confirm the presence of NMBCs 1–5 in ER+ human breast tumours and NMBC 6 in ER– tumours (Samuel et al. 1997*b*).

Using the cross-linking agent cisplatin, we found that most, but not all, abundant nuclear matrix bound proteins are also bound to DNA in situ (Davie et al. 1998; Samuel et al. 1998). We are currently using this cross-linking agent to identify informative breast cancer nuclear matrix proteins that are associated with nuclear DNA in situ.

Transcription factors and the nuclear matrix

Transcription factors are associated with the nuclear matrix (Davie 1997; Stein et al. 1997). Recent studies have identified nuclear matrix targeting sequences for several transcription factors, including YY1, AML, ER, and Pit-1 (Stenoien et al. 1998; Zeng et al. 1998; McNeil et al. 1998; Mancini et al. 1999). It has been proposed that the nuclear matrix recruits transcription factors, facilitating their interaction with regulatory DNA elements (Zeng et al. 1998). We applied the cisplatin cross-linking procedure to investigate whether nuclear matrix-associated transcription factors were bound to nuclear matrix attached DNA in situ in ER+, hormone-dependent MCF-7 human breast cancer cells (Samuel et al. 1998). The nuclear matrix-associated transcription factors studied were: ER, a transcription factor essential to the proliferation of hormone-dependent breast cancer cells; hnRNP K, a single-strand DNA-binding transcription factor; and HET/SAF-B, a MAR-binding protein that acts as a repressor of hsp27 gene expression (Oesterreich et al. 1997). We found that these nuclear matrix-bound transcription factors were cross-linked to MAR DNA in situ. In contrast, a nuclear matrix protein, SRm160, involved in RNA splicing (Blencowe et al. 1998) was not cross-linked to DNA (Samuel et al. 1998). These observations provide the first direct evidence that nuclear matrix bound transcription factors are also bound to MARs in situ. Further, the results provide evidence that the nuclear matrix is not simply a storage site for inactive transcription factors. Clearly, the nuclear matrix-associated transcription factors are functional in the sense that they are bound to nuclear DNA sequences.

Histone deacetylase, gene repression and cancer

Mammalian histone deacetylases, HDAC1, HDAC2, HDAC3, and the recently reported HDA (Verdel and

Khochbin 1999), are components of large multiprotein complexes (Davie and Chadee 1998). For example, mammalian HDAC1 and HDAC2 are in large multiprotein complexes containing mSin3, N-CoR, or SMRT (corepressors), SAP18, SAP30, RbAp48, RbAp46, and c-Ski (Nomura et al. 1999). Recruitment of the HDAC complex to a specific regulatory element results in repression of gene expression. Repression may occur by one of several mechanisms, including deacetylation of histones resulting in a condensed chromatin structure, deacetylation of nonhistone chromosomal proteins or transcription factors, and disruption of a functional preinitiation complex (Davie and Chadee 1998). Several signalling pathways regulate the recruitment of the HDAC corepressor complex to specific loci. The Sin3A-N-CoR-HDAC1,2 complex, for example, is recruited by unliganded nuclear receptors and the Mad family of basic helix-loop-helix-zipper proteins (Davie and Chadee 1998).

Fos-transformed cells have elevated levels of DNA 5-methylcytosine transferase. *Fos* transformation can be reversed by inhibiting the activity of DNA 5-methylcytosine transferase or histone deacetylases (Bakin and Curran 1999). The discovery that methyl-CpG-binding protein 2 (MeCP2) binds to Sin3, recruiting the HDAC (1 and 2) multiprotein complex, provided evidence that DNA methylation and histone deacetylation are coupled events in the formation of repressive chromatin structures and gene silencing (Nan et al. 1998; Jones et al. 1998). In some situations, however, inhibition of histone deacetylases will not reverse the repression of methylated promoters (Cameron et al. 1999). For such loci, dense CpG island methylation appears to be dominant in silencing. Alternatively, these regions may not be associated with histone acetyltransferases, which would acetylate the histones when histone deacetylase activity is inhibited.

Recently, a new HDAC complex, called NuRD, consisting of N-CoR, MTA2 (highly related to metastasis-associated protein MTA1), Mi2, and RbAP46/48 was isolated. NuRD (nucleosome remodelling histone deacetylase complex) has both ATP-dependent chromatin remodelling and histone deacetylase activities (Tong et al. 1998; Wade et al. 1998; Zhang et al. 1998; Xue et al. 1998). ATP stimulation of deacetylation of chromatin templates by NuRD varied from no stimulation to about threefold.

Oncoproteins, PML-RARα, PLZF-RARα, and AML-1-ETO, expressed in acute promyelocytic and myeloid leukemia recruit SMRT (N-CoR)-mSin3A-HDAC1 complexes (Lin et al. 1998; Fenrick and Hiebert 1998; Grignani et al. 1998; Lutterbach et al. 1998). The recruitment of HDAC1 is crucial to the transforming potential of these oncoproteins. Inhibiting the HDAC activity with new generation HDAC inhibitors appears to be a promising approach to the treatment of these cancers (Lin et al. 1998; Richon et al. 1998; Nakajima et al. 1998; Saunders et al. 1999).

Histone deacetylase and organization of nuclear DNA

In our studies investigating whether nuclear matrix-associated transcription factors were bound to nuclear matrix-attached DNA in situ in MCF-7 human breast cancer cells, we found that HDAC1 was bound to MARs in situ (Samuel et al. 1998). Further, using the cross-linking agent cisplatin, we observed that chicken nuclear matrix bound HDAC1 was associated with MAR DNA in G0 phase erythroid cells (Sun et al. 1999). These results suggest that HDAC1 is associated with MARs at the base of loops. A possible mechanism for the recruitment of HDAC1 to MARs is through the attachment region binding protein (ARBP), a nuclear matrix-associated MAR-binding protein. Strätling and colleagues found that ARBP is homologous to MeCP2 (Weitzel et al. 1997). Thus, MARs associated with ARBP/MeCP2 could recruit the HDAC1 complex to the base of chromatin loops (Fig. 2). The recruitment of the HDAC multiprotein complex to the base of chromatin loops has interesting possibilities in chromatin architecture. Crane-Robinson and colleagues reported that at the boundaries of the DNase I-sensitive, transcriptionally competent β-globin chromatin domain of chicken erythrocytes there is a marked change in the acetylation state of the histones (Hebbes et al. 1994). The β-globin chromatin domain is associated with highly acetylated histones, while histones at the boundaries are poorly acetylated. Several studies have shown that the boundaries of DNase I sensitive domain co-map with MARs (Davie 1995). The association of nuclear matrix-bound HDAC1 with MARs would provide a mechanism by which the histones at the boundaries of the domain are deacetylated.

Our studies have shown that HDAC1 is associated with avian and human nuclear matrices (Samuel et al. 1998; Sun et al. 1999). Besides ARBP/MeCP2, there are several other nuclear matrix-associated transcription factors that could recruit the HDAC1 to specific nuclear sites. The retinoblastoma protein, a tumour suppressor, is associated with nuclear matrix and HDAC1, but only when in a hypophosphorylated state (Mancini et al. 1994; Luo et al. 1998; Brehm et al. 1998; Magnaghi-Jaulin et al. 1998). YY1 is another nuclear matrix protein that is associated with HDAC1 (Yang et al. 1997). It will be interesting to determine how mechanical and chemical signalling pathways affect the nuclear location and activity of HDAC1. A recent study gives us a glimpse of the role of histone deacetylase in nuclear organization and cell function (Lelièvre et al. 1998). These authors showed that inhibition of histone deacetylase activity in tissue-like acini formed from human mammary epithelial cells disrupted the nuclear organization of NuMA and altered the acinar phenotype as evidenced by the loss of endogenous basement membrane.

Summary and future directions

We have come to appreciate the compartmentalization of the components within the interphase nucleus, giving rise to a highly organized environment. Specific nuclear domains involved in transcription, replication, splicing, and storage have been identified (Schul et al. 1998; Hodges et al. 1998; Misteli and Spector 1998; Wei et al. 1998). The dynamic nuclear matrix has a role in mediating this high level of spatial and functional organization within the nucleus. Protein machines or transcription factories involved in processing the genetic information are assembled on the nuclear matrix (Nickerson 1998; Hendzel et al. 1998; Wei et al. 1998; Jackson et al. 1998). Both mechanical and chemical signalling

pathways affect the structural organization of the nucleus. In malignant cells these signalling pathways are deregulated, perturbing nuclear architecture (Nickerson 1998; Boudreau and Bissell 1998). Alterations in nuclear matrix and chromatin structure can result in aberrant gene expression and genetic instability (Pienta and Ward 1994; Nickerson 1998). Cancer-specific nuclear matrix proteins hold promise as biomarkers in the diagnosis and prognosis of cancer (Hughes and Cohen 1999). Development of drugs that target nuclear matrix proteins or inhibit chromatin modifying enzymes, some of which are downstream targets of the signalling pathways, is a promising approach in the treatment of cancer (Nickerson 1998; Fenrick and Hiebert 1998; Warrell et al. 1998). Thus, it is an important goal to identify and characterize histone-modifying enzymes (e.g., histone H3 kinase) that are activated by signalling pathways.

Acknowledgements

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Direct Visualization of the Human Estrogen Receptor α Reveals a Role for Ligand in the Nuclear Distribution of the Receptor

Han Htun,*†‡ Laurel T. Holth,‡§ Dawn Walker,* James R. Davie,§ and Gordon L. Hager*‖

*Laboratory of Receptor Biology and Gene Expression, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; and §Department of Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3

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The human estrogen receptor α (ER α) has been tagged at its amino terminus with the S65T variant of the green fluorescent protein (GFP), allowing subcellular trafficking and localization to be observed in living cells by fluorescence microscopy. The tagged receptor, GFP-ER, is functional as a ligand-dependent transcription factor, responds to both agonist and antagonist ligands, and can associate with the nuclear matrix. Its cellular localization was analyzed in four human breast cancer epithelial cell lines, two ER+ (MCF7 and T47D) and two ER− (MDA-MB-231 and MDA-MB-435A), under a variety of ligand conditions. In all cell lines, GFP-ER is observed only in the nucleus in the absence of ligand. Upon the addition of agonist or antagonist ligand, a dramatic redistribution of GFP-ER from a reticular to punctate pattern occurs within the nucleus. In addition, the full antagonist ICI 182780 alters the nucleocytoplasmic compartmentalization of the receptor and causes partial accumulation in the cytoplasm in a process requiring continued protein synthesis. GFP-ER localization varies between cells, despite being cultured and treated in a similar manner. Analysis of the nuclear fluorescence intensity for variation in its frequency distribution helped establish localization patterns characteristic of cell line and ligand. During the course of this study, localization of GFP-ER to the nucleolar region is observed for ER− but not ER+ human breast cancer epithelial cell lines. Finally, our work provides a visual description of the "unoccupied" and ligand-bound receptor and is discussed in the context of the role of ligand in modulating receptor activity.

INTRODUCTION

Steroid hormones elicit diverse biological responses, important during growth, differentiation, inflammation, pregnancy, and homeostasis among many other processes. The genomic actions of steroid hormones are mediated by steroid receptors, members of the nuclear receptor superfamily of ligand-dependent transcription factors. In the absence of hormone, steroid receptors exist in a complex with chaperone proteins capable of high-affinity binding to steroid hormones. Hormone binding leads to a conformational change in the receptor that results in its dissociation from chaperone proteins and ultimately in the binding of the receptor as a homodimer to cognate sites in steroid-responsive genes (reviewed in Tsai and O'Malley, 1994; Mangelsdorf *et al.*, 1995; Beato *et al.*, 1996).

Immunohistochemistry and biochemical fractionation show the unoccupied steroid receptors to reside

‡ These authors contributed equally to this work.
‖ Corresponding author. E-mail address: hagerg@exchange.nih.gov.
† Present address: Departments of Obstetrics and Gynecology and Molecular and Medical Pharmacology, 27-139 CHS, University of California Los Angeles School of Medicine, 10833 Le Conte Avenue, Los Angeles, CA 90095-1740.

predominantly in the cytoplasm, the nucleus, or both compartments, depending on the receptor, in a complex with chaperone proteins (Jensen, 1991; DeFranco *et al.*, 1995; Beato *et al.*, 1996; Pratt and Toft, 1997). For the predominantly nuclear receptors, such as the estrogen receptor (ER), the unoccupied receptor exists in the nucleus either bound or not bound to its cognate site in target genes. Hormone binding leads to activation of the receptor and transcriptional regulation of the responsive genes (Press *et al.*, 1989; Picard *et al.*, 1990; Parker, 1992; Tsai and O'Malley, 1994).

We and others have previously shown that the subcellular localization and trafficking of the glucocorticoid receptor (GR) can be followed with a green fluorescent protein (GFP) fusion (Ogawa *et al.*, 1995; Carey *et al.*, 1996; Htun *et al.*, 1996; Rizzuto *et al.*, 1996). The chimeric receptor GFP-GR can be fully functional as a ligand-dependent transcription factor and shows the ligand specificity of GR. The ligand-dependent translocation of GFP-GR from the cytoplasm to nucleus can be observed in real time in a single cell. Most interestingly, we observed an intranuclear pattern and distribution of GFP-GR that reflects the type of ligand, either agonist or antagonist, used to activate the receptor (Htun *et al.*, 1996).

In this report, we have chosen a similar strategy with the human ER α, referred to throughout the paper as ER, to see whether ligand affects the nuclear distribution of this receptor. Previous biochemical studies described the existence of two biochemically distinct forms of ER (Gorski *et al.*, 1968; Jensen *et al.*, 1968). In the absence of ligand, the "unoccupied" ER with a sedimentation coefficent of 9S is "loosely" associated with the nucleus; ligand causes a biochemical transformation to a complex with a sedimentation of 5S that associates more "tightly" with the nucleus (Greene and Press, 1986; Press *et al.*, 1989; Jensen, 1991). To determine whether the biochemical difference is reflected by a change in the intranuclear distribution of ER, we have directly visualized ER in living cells by tagging the receptor with the S65T variant of the naturally fluorescent protein GFP.

MATERIALS AND METHODS

Cell Lines and Plasmids

Human breast cancer epithelial cell lines MCF-7, T47D, and MDA-MB-231 were obtained from American Type Culture Collection (Rockville, MD). MDA-MB-435A is a derivative of MDA-MB-435 (Yee *et al.*, 1996). Unless otherwise noted, cells were maintained in a 225-cm^2 cell culture flask (Costar, Cambridge, MA) in 35 ml of Dulbecco's modified Eagle's medium (DMEM) with phenol red as pH indicator (Life Technologies, Grand Island, NY; catalog number 11960-044), supplemented with 10% FBS (Life Technologies; catalog number 10437-028), 2 mM L-glutamine, 4.8 μg/ml insulin, and 100 U of penicillin G/100 μg of streptomycin sulfate/ml at 37°C in a 5% CO_2-water jacketed incubator. Medium was changed every 2 d. At confluence, cells were harvested by first washing with Dulbecco's PBS (D-PBS) without calcium or magnesium and then treating with 0.05% trypsin-0.02% EDTA without phenol red. Cells were reseeded in a fresh flask at about a one-to-four dilution. Four days before transfection, cells were placed in DMEM lacking phenol red (Life Technologies; catalog number 31053-028) and supplemented as described above, except dextran/charcoal–treated FBS (Hyclone, Logan, UT; catalog number SH30068.03) was used in place of FBS. The reporter gene, pERE-tk-CAT, contains two copies of a perfect estrogen response element (ERE) in tandem, upstream of the thymidine kinase (tk) minimal promoter hooked up to the chloramphenicol acetyltransferase (CAT) reporter gene (Seiler-Tuyns *et al.*, 1986). The human ER expression vector pSG5-HEGO (Tora *et al.*, 1989), contains a wild-type human ER under the control of the SV40 early promoter. The GFP-ER expression plasmid pCI-nGL1-HEGO (GenBank database; accession number AF061181), was prepared by first replacing the S65T GFP coding region in the plasmid pCI-nGFP-C656G (Htun *et al.*, 1996) with an S65T GFP coding region optimized for expression in mammalian cells from pGreen Lantern-1 (Life Technologies) and then replacing the rat GR with the human ER coding region in pSG5-HEGO, previously mutated with the Chameleon site-directed mutagenesis kit (Stratagene, La Jolla, CA) to introduce an *MluI* site in the first three amino acids of the ER coding region with the oligonucleotide 5′-TGGTGTGGAGGGTCAACGCGT-TGGTCCGTGGCCGCG-3′. To enrich for cells transfected with the expression plasmids, the plasmid pCMV-IL2R, which expresses the human interleukin 2 receptor (IL2R), was used in the transfection experiments, as previously described (Htun *et al.*, 1996).

Transfections

Plasmid DNA was transiently introduced into cells either by calcium phosphate coprecipitation, electroporation, or liposome-mediated gene transfer. For ERE reporter assays, MDA-MB-435 cells were plated in 100-mm dishes in DMEM lacking phenol red with 5% twice charcoal-stripped FBS 2 d before transfection and were given fresh media 24 h before transfection. Cells were maintained at 37°C in a 5% CO_2 incubator before transfection. Cells were transfected by the $CaPO_4$/BES precipitation method (Kingston *et al.*, 1995). One ml of precipitate contained 4 μg of pCH110 (β-galactosidase reporter plasmid) as an internal control, 5 μg of ERE-tk-CAT reporter plasmid, pCI-nGL1-HEGO in the amount indicated, and pCEP4 as carrier DNA to a total of 15 μg of DNA. Cells were in contact with precipitate for 14 h and then washed twice with D-PBS and treated with fresh phenol red–free media plus 5% twice charcoal-stripped FBS containing 17β-estradiol, ICI 182780, or 4-hydroxytamoxifen as indicated. Cells were harvested 30 h after the removal of the calcium phosphate precipitates. All transfections were done in triplicate. CAT assays (Kingston *et al.*, 1995) and β-galactosidase assays (Sambrook *et al.*, 1989) were done using the method essentially as described. CAT activity was normalized to β-galactosidase activity, and the mean and SEM for three sets of data were plotted. For electroporation, cells were electroporated with the indicated amount of pCI-nGL1-HEGO DNA with or without 5 μg of pCMV-IL2R DNA for 2×10^7 cells in 0.2 ml of cold phenol red–free DMEM at 250 V and 1100 μF in a 0.4-cm electrode gap electroporation chamber supplied with the Cell-Porator electroporation system (Life Technologies; catalog number 71600-019), left to recover on ice for 5 min, and then diluted in phenol red–free DMEM supplemented with 10% dextran/charcoal–treated FBS before plating. Cells were then grown for 12–16 h in 37°C, 5% CO_2 incubators before imaging. Liposome-mediated gene transfer was used as directed by the manufacturer of DOSPER liposomal transfection reagent (Boehringer Mannheim, Indianapolis, IN; catalog number 1 811 169) for nuclear matrix isolation experiments.

Enrichment of Transfected Cells and Analysis of Total Cell Lysates with Human ER Monoclonal Antibody H226

Approximately 18 h after electroporation with 5 μg of pCMV-IL2R and various amounts of pCI-nGL1-HEGO, cells were washed twice

with PBS and then sorted with magnetic beads coated with antibody to the IL2R, as previously described (Htun *et al.*, 1996). IL2R-positive cells were washed several times and then divided into two equal aliquots. One aliquot was lysed with an NP-40 lysis solution (0.5% NP-40, 50 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, pH 7.5, and a mixture of protease inhibitors) after incubating on ice for 5 min and then centrifuged for 5 min in a microfuge to remove the insoluble debris. The protein concentration in the soluble extract was determined by the Bradford method (Bradford, 1976) using a Bio-Rad (Hercules, CA) protein assay reagent. The other half of the cells was used to prepare total cellular lysate with 1× SDS gel-loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol; Sambrook *et al.*, 1989) in the same volume as the NP-40 lysis solution. Based on the protein concentration, a volume equivalent to 30–40 μg was removed from the total cellular lysate, heated to 90°C for 5 min, subjected to denaturing polyacrylamide gel electroporesis in 8% polyacrylamide (30 acrylamide:1 bisacrylamide) containing 0.1% SDS, and analyzed by the Western blotting method, essentially as described previously (Sambrook *et al.*, 1989), using a rat monoclonal antibody, H226, raised against the human ER α (a gift from Geoffrey Greene, The Ben May Institute for Cancer Research, University of Chicago, Chicago, IL) as a primary antibody and donkey anti-rat antibody conjugated to horseradish peroxidase as a secondary antibody with the Pierce (Rockford, IL) Super Signal chemiluminescent substrate.

Extraction of Nuclear Matrix

A modified procedure was used for the extraction of nuclear matrix (Jackson and Cook, 1988; Belgrader *et al.*, 1991; Berezney, 1991). Nuclei from frozen MCF-7 cell pellets, which had been transiently transfected with 15 μg of pCI-nGL1-HEGO HEGO in 90 μl of DOSPER liposomal transfection reagent for 1×10^6 cells and grown with DMEM in CSFBS without phenol red, were resuspended in TNM buffer (10 mM Tris, pH 8.0, 300 mM sucrose, 100 mM NaCl, 2 mM $MgCl_2$, 1%, vol/vol, thiodiglycol, and 1 mM PMSF) and homogenized three times in a Dounce homogenizer with a Teflon pestle. Triton X-100 was added to a final concentration of 0.5%, vol/vol, and nuclei were collected by centrifugation at 500 × *g* for 10 min at 4°C. Nuclei were then resuspended in TNM buffer, homogenized, and pelleted as described above. The isolated nuclei were then resuspended to a concentration of 20 A_{260}/ml in DIG buffer (10 mM Tris, pH 8.0, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 1%, vol/vol, thiodiglycol, and 1 mM PMSF) and digested with 168 U/ml DNase I (D5025; Sigma, St. Louis, MO) for 20 min at room temperature. Ammonium sulfate was added to a final concentration of 0.25 M, and nuclear matrix was collected by centrifugation. The pellet NM-IF1 was then resuspended in DIG buffer, followed by the addition of an equal volume of 4 M NaCl. The nuclear matrix was then collected by centrifugation and re-extracted with 2 M NaCl as above. The final pellet NM-IF2 was resuspended in 1× SDS gel-loading buffer, as previously described. Supernatants from the 0.25 M ammonium sulfate and 2 M NaCl extractions were dialyzed overnight against double-distilled H_2O and 1 mM PMSF and then lyophilized. All four samples were subjected to denaturing polyacrylamide gel and Western blot analyses, essentially as described above, using an antibody (BabCo, Berkeley, CA) against the hemagglutinin (HA) tag (YPYDVPDYA) at the amino terminus of GFP-ER.

Microscopy

For differential interference contrast, the cells electroporated with only pCI-nGL1-HEGO were grown on glass coverslips overnight. The coverslips were rinsed with D-PBS and placed inverted on a microscope slide. Cells were illuminated by white light from a tungsten light source and viewed under a 63×, 1.4 numerical aperture Plan-Apochromat oil immersion objective in a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY) under Nomarski optics. For green fluorescence, the same cells were examined through a 480- to 490-nm excitation, a 510-nm dichroic mirror, and a 515- to 565-nm emission filter using a 100-W mercury bulb light source. Images were recorded on Kodak (Rochester, NY) Elite 200 color slide film. In the case of confocal laser scanning microscopy, cells were imaged through a 100×, 1.4 numerical aperture Plan-Apochromat oil immersion objective by excitation with the 488-nm line from a krypton–argon laser, and the emission was viewed through a 506- to 538-nm band pass filter. Images were collected on a Zeiss Axiovert 135 platform attached to a Bio-Rad MRC 1024 confocal imaging system using Bio-Rad LaserSharp software.

Image Analysis

Image analyses and representation in Figure 2 were performed on an Apple (Cupertino, CA) Power Macintosh 8600/200 computer using the public domain NIH Image program version 1.61 (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). To generate Table 1, additional image analyses were performed by determining mean pixel value and SE for each area containing a nucleus but excluding the nucleoli using IPLab Spectrum software (Signal Analytics, Fairfax, VA) operating on a Power Macintosh 8600/200 computer. To obtain the coefficient of variation for fluorescence intensity, the SD of the pixel values for each nucleus was divided by the mean pixel value. The mean of the coefficient of variation for the population and the SD were determined for each set of cells exposed to the same ligand and are provided in Table 1. Statistical significance was determined using the Z test (Chase and Bown, 1992).

RESULTS

Expression and Functional Analysis of the GFP-ER Fusion Protein

To follow the subcellular localization and trafficking of the ER in living cells, we tagged the amino terminus of the human ER α (ER) with the S65T variant of GFP. Figure 1A shows the cytomegalovirus (CMV) enhancer/promoter–driven GFP-ER expression vector. Functionality of GFP-ER as a ligand-dependent transcription factor was assayed on an estrogen response element–containing reporter gene in transient transfection experiments in a human breast cancer epithelial cell line, MDA-MB-435A, which lacks endogenous ER (Yee *et al.*, 1996). GFP-ER activates the reporter gene in a dose-dependent manner and shows additional activation in the presence of agonist ligand 17β-estradiol (Figure 1C). Significant ligand-independent activation has previously been reported for the ER (Danielian *et al.*, 1992). Similar results were obtained with the unsubstituted ER (our unpublished results). Maximal activation of GFP-ER by 17β-estradiol was observed at a 10 nM concentration of ligand (Figure 1D; lanes 1–5), consistent with the previously reported subnanomolar K_d for ER (Kuiper *et al.*, 1997). ICI 182780, a pure antagonist for ER (Wakeling *et al.*, 1991), completely inhibited GFP-ER activation of the reporter gene (Figure 1D, compare lanes 1 and 6). When ER antagonists were present in 25- to 50-fold molar excess, the action of 17β-estradiol was inhibited, albeit the pure antagonist ICI 182780 compound was more effective than the partial antagonist 4-hydroxytamoxifen (Figure 1D, compare lanes 4 and 8 and lanes 4

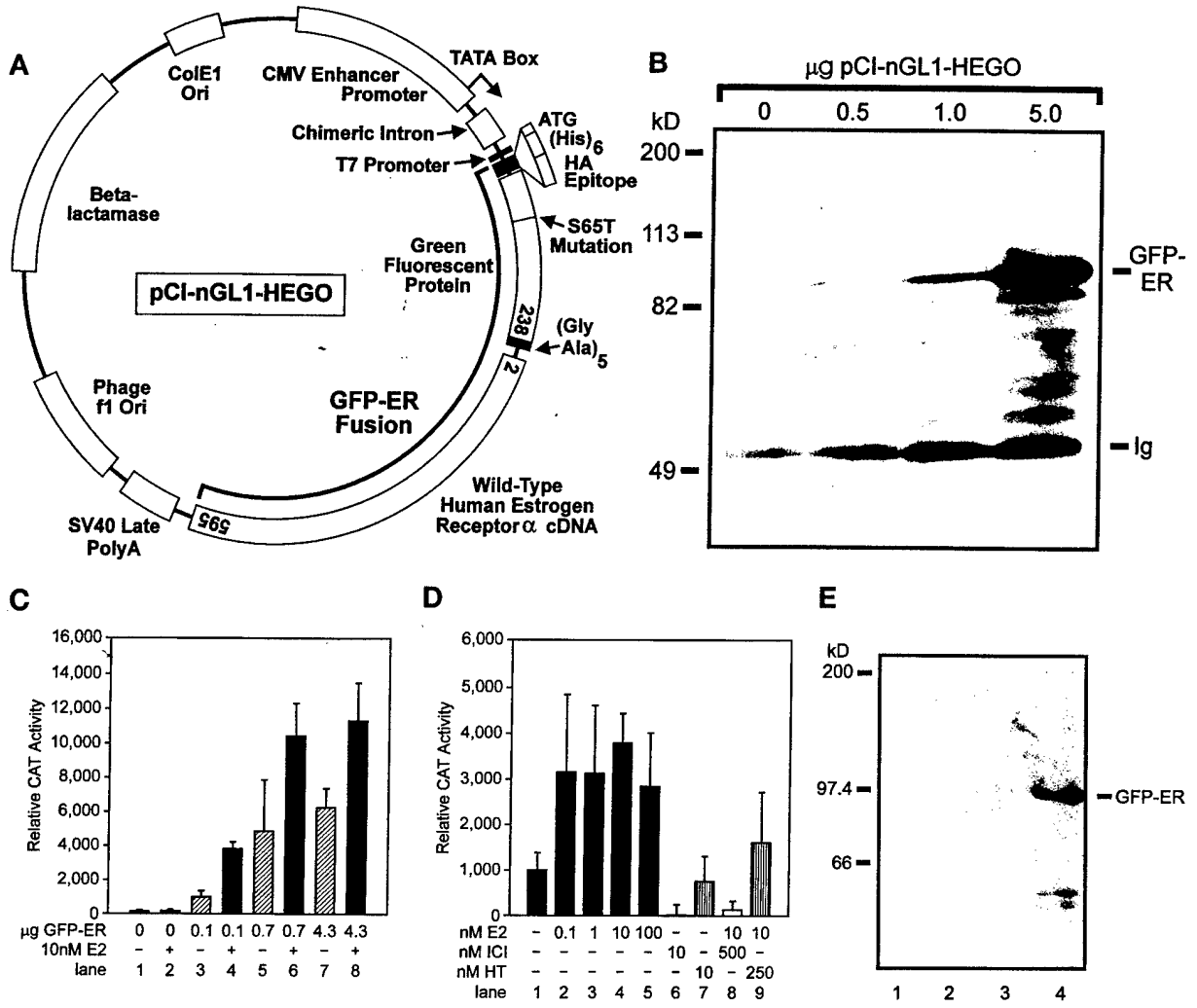

Figure 1. Construction and characterization of GFP-ER. (A) Map of GFP-ER expression plasmid pCI-nGL1-HEGO. The human wild-type ER is fused to the carboxyl terminus of an S65T variant of GFP whose codons have been optimized for translation in mammalian cells. A small linker region containing five glycine-alanine repeats separates the two coding regions. The S65T GFP is tagged with a $(his)_6$ and HA epitope at the amino terminus. Amino acids present in the fusion protein are indicated in the open boxes for GFP and ER. (B) Expression of GFP-ER in MCF-7 cells. MCF-7 cells were electroporated with the indicated amount of GFP-ER expression plasmid pCI-nGFL1-HEGO and 5 μg of IL2R expression plasmid pCMV-IL2R and allowed to express for 16 h. The population of transiently transfected cells was identified by the presence of the IL2R cell surface marker and isolated by magnetic beads coated with the anti-IL2R monoclonal antibody, as previously described (Htun *et al.*, 1996). The total cellular lysate prepared from these cells was analyzed for the presence of GFP-ER by Western blot analysis using a rat monoclonal antibody, H226, against human ER as primary antibody, donkey anti-rat antibody coupled to horseradish peroxidase as second antibody, and Pierce Super Signal chemiluminescent substrate. Location of GFP-ER is indicated along with that of the Ig, which is present in the extract because of the cell-sorting procedure and is also detected by the secondary antibody. Molecular weight markers are indicated on the left. (C) GFP-ER–dependent and 17β-estradiol–dependent transcriptional activation of the ERE-containing reporter gene pERE-tk-CAT in MDA-MB-435A cells. Human breast cancer epithelial cells, MDA-MB-435A, were transfected with various amounts of the GFP-ER expression plasmid as indicated along with 5 μg of pERE-tk-CAT reporter gene and 4 μg of β-galactosidase expression plasmid pCH110 by the calcium phosphate coprecipitation method. After replacement with fresh medium the next day, cells were treated (gray bars) or not treated (stippled bars) with 10 nM 17β-estradiol for 30 h. Extracts were prepared subsequently and assayed for CAT and β-galactosidase activity. The relative CAT activity was calculated using the β-galactosidase activity to normalize for transfection efficiency. (D) Effect of 17β-estradiol concentration and type of ligand on GFP-ER activation of the pERE-tk-CAT reporter gene in MDA-MB-435A cells. Cells were transfected as described in C using 0.1 μg of pCI-nGL1-HEGO expression plasmid, 5 μg of pERE-tk-CAT reporter gene, and 4 μg of β-galactosidase expression plasmid pCH110. After transfection, cells were treated with no additional ligand (lane 1), 0.1 nM 17β-estradiol (lane 2), 1 nM 17β-estradiol (lane 3), 10 nM 17β-estradiol (lane 4), 100 nM 17β-estradiol (lane 5), 10 nM ICI 182780 (lane 6), 10 nM 4-hydroxytamoxifen (lane 7), 500 nM ICI 182780 and 10 nM 17β-estradiol (lane 8), and 250 nM 4-hydroxytamoxifen and 10 nM 17β-estradiol (lane 9) for 30 h before analysis. (E) Association of GFP-ER with the nuclear matrix in MCF-7 cells. MCF-7 cells were grown in charcoal-stripped FBS. Lane 1, supernatant after extraction of isolated nuclei with 0.25 M ammonium sulfate; lane 2, supernatant after the first extraction with 2M NaCl; lane 3, supernatant after the second extraction with 2 M NaCl; lane 4, nuclear matrix and associated intermediate filaments. Immunodetection of GFP-ER was done using the HA antibody to the HA tag located on the N terminus of the GFP-ER fusion protein.

and 9). Thus, GFP-ER functions as a transcriptional activator, and its activity is fully regulated by ER ligands.

Presence of the GFP tag results in a receptor that is ~27 kDa larger than the untagged ER. An immunoblot using a rat monoclonal ER antibody, H226, of total cellular lysates prepared from MCF-7 cells transfected with GFP-ER expression vector pCI-nGL1-HEGO shows the presence of a protein with a molecular mass of ~94 kDa, the expected molecular mass of the fusion protein (Figure 1B). Furthermore, from the width and intensity of the band, inclusion of a greater amount of GFP-ER expression plasmid in a transfection results in more fusion protein being expressed in the transfected cells.

Previous investigations have shown that unliganded ER associates loosely with the nucleus but that the liganded ER associates more tightly with the nucleus (Greene and Press, 1986; Press *et al.*, 1989; Jensen, 1991). Several studies have also suggested that steroid receptors are associated with the nuclear matrix (Barrack and Coffey, 1980; Alexander *et al.*, 1987; Samuel *et al.*, 1998). In addition, transcribing chromatin has been reported to be selectively associated with the nuclear matrix (Davie, 1995; Davie *et al.*, 1997). These considerations led us to examine the potential association of GFP-ER with the matrix. Nuclear matrix was prepared from MCF-7 cells transfected with the GFP-ER expression plasmid. Figure 1E shows the result of two successive 2 M NaCl extractions of a nuclear matrix preparation from MCF-7 cells whose nuclei have been digested with DNase I and extracted with 0.25 M ammonium sulfate. From the Western blot analysis using an anti-HA antibody to detect the HA epitope at the amino terminus of GFP-ER, the GFP-ER that remains associated with the initial nuclear matrix preparation is tightly bound to the matrix and resistant to 2 M NaCl extraction (Figure 1E, compare lanes 1–3 with lane 4). The initial cytosolic fraction contains soluble nuclear protein and significant amounts of GFP-ER (our unpublished results). Comparable results were obtained when cells were grown in the presence of estrogens (our unpublished results). These findings are consistent with the earlier description of ER as cytosolic, based on biochemical fractionation experiments (Gorski *et al.*, 1968; Jensen *et al.*, 1968; Greene and Press, 1986; Press *et al.*, 1989; Jensen, 1991), as well as a nuclear matrix-binding protein (Barrack and Coffey, 1980; Alexander *et al.*, 1987).

Cellular Localization of GFP-ER

To determine the localization of the tagged ER, we examined various human breast cancer epithelial cell lines transfected with the GFP-ER expression vector. In particular, we were interested in the effect of ligand as well as cellular structure and milieu on GFP-ER subcellular localization in the ER-positive (ER+) and ER-negative (ER−) cell lines. Four representative human breast cancer epithelial cell lines were examined, two ER+ (MCF-7 and T47D) and two ER− (MDA-MB-231 and MDA-MB-435A). Green fluorescence can be detected by conventional fluorescence microscopy in MCF-7 cells after electroporation with the GFP-ER expression plasmid, indicating that the GFP chromophore in the fusion protein is functional (Figure 2B). Comparison with the differential interference contrast image shows green fluorescence to be restricted to the nucleus of a few cells successfully transfected with the GFP-ER expression plasmid (Figure 2A, white arrows, also compare A and B). In the cells with a lower level of green fluorescence, nucleolar outlines are observed, which is consistent with GFP-ER being excluded from the nucleolus (Figure 2B, two leftmost nuclei and three lower nuclei). Thus, GFP-ER localization is consistent with that previously reported for the endogenous ER in MCF-7 cells (King and Greene, 1984; Welshons *et al.*, 1988).

Inclusion of 10 nM ligand, either agonist 17β-estradiol (Figure 2, C and D) or partial antagonist 4-hydroxytamoxifen (Figure 2, E and F), during the time of transfection and culturing of the cells for 20 h had no apparent effect on the nucleocytoplasmic compartmentalization of GFP-ER. In contrast, when GFP-ER–expressing cells were treated with 10 nM ICI 182780, a pure ER antagonist, green fluorescence was observed not only in the nucleus but also in the cytoplasm (Figure 2, G and H). This effect of ICI 182780 on the nucleocytoplasmic compartmentalization of GFP-ER is similar to that previously reported for the untagged ER (Dauvois *et al.*, 1993).

Although a similar effect of ligand on GFP-ER nucleocytoplasmic compartmentalization was observed in T47D, MDA-MB-231, and MDA-MB-435A cells (our unpublished results), we observed variability in the proportion of cells showing cytoplasmic green fluorescence after overnight treatment with 10 nM ICI 182780. In T47D and MCF-7 cells, cytoplasmic green fluorescence was seen in a minority of the cells within 30 min of 10 nM ICI 182780 treatment and peaked at 6–8 h with ~90% of the cells showing some degree of cytoplasmic fluorescence (e.g., Figure 3B). In the case of the ER− cell lines, cytoplasmic accumulation was observed in ~10% of the population despite prolonged treatment (20 h) with 10 nM ICI 182780 (our unpublished results). However, in no case did total cytoplasmic green fluorescence exceed nuclear fluorescence (e.g., Figures 2H and 3B).

Effect of Cycloheximide on ICI 182780-induced Cytoplasmic Accumulation of GFP-ER

The protein synthesis inhibitor cycloheximide was used to block protein synthesis, and its effect on cytoplasmic accumulation of GFP-ER was examined after

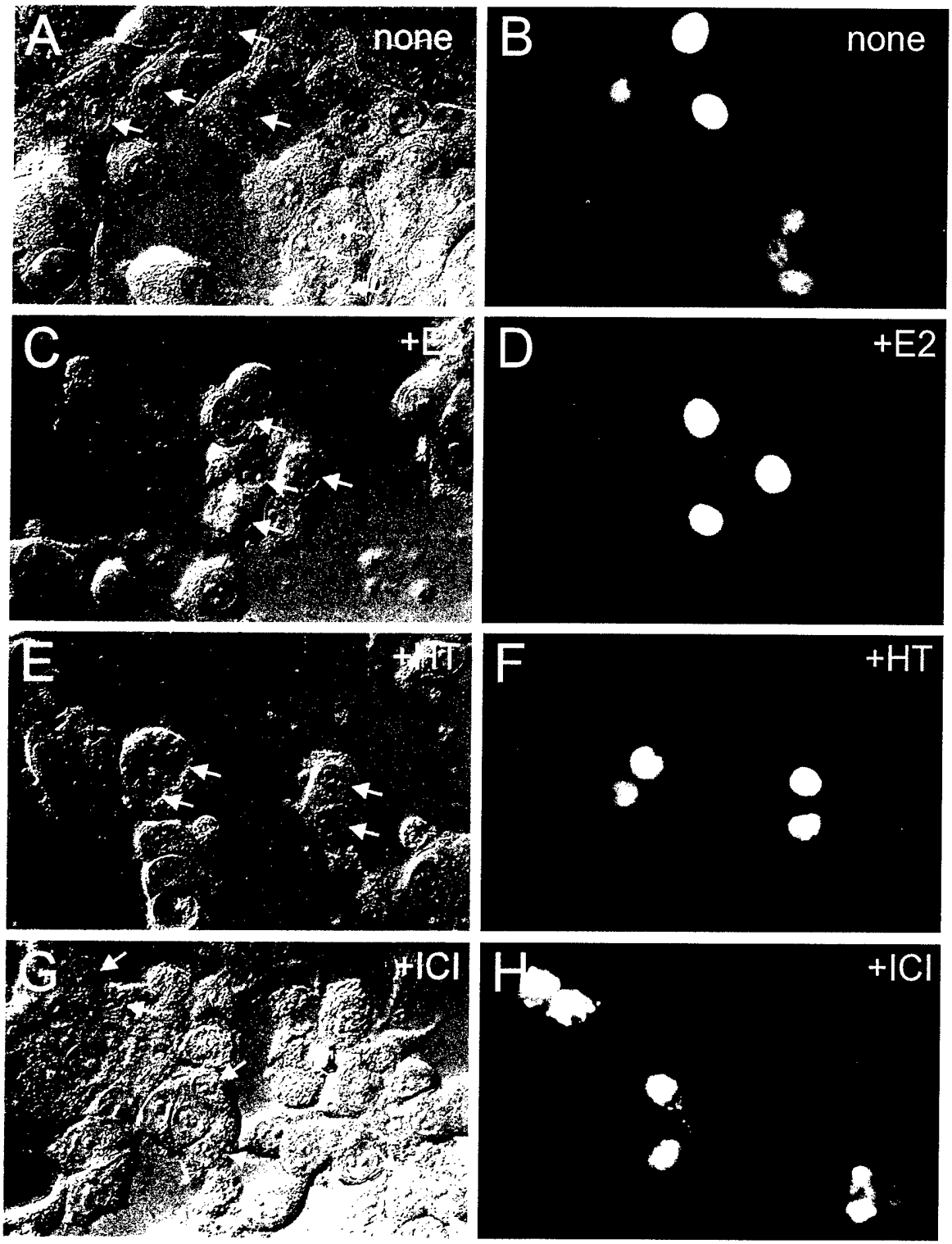

Figure 2. Effect of ligand treatment on GFP-ER localization in MCF-7 cells. Cells from a human breast cancer epithelial cell line, MCF-7, were electroporated with 0.2 μg of GFP-ER expression plasmid, pCI-nGL1-HEGO, and cultured on coverslips for 12–16 h before visualization by differential interference contrast (A, C, E, and G) or epifluorescence with a standard set of FITC filters (B, D, F, and H). Cells were treated at the time of culturing with nothing (A and B), 10 nM 17β-estradiol (C and D), 10 nM 4-hydroxytamoxifen (E and F), or 10 nM ICI 182780 (G and H). The arrow in the left panel indicates the nucleus of a cell exhibiting green fluorescence, as seen in the right panel.

10 nM ICI 182780 treatment. In MCF-7 cells treated with 200 μg/ml cycloheximide for 20 h immediately after electroporation, no green fluorescence was seen (compare Figure 3, C and D, with no cycloheximide treated cells in Figure 2, A and B), indicating that no GFP-ER was synthesized in the cycloheximide-treated cells. In contrast, allowing protein synthesis to continue for the first 12 h after transfection and then halting it for 8 h through the addition of cycloheximide to the culture medium showed green fluorescence in the nucleoplasm but not in the nucleolus (Figure 3, E and F). Simultaneous inclusion of both cycloheximide and 10 nM ICI 182780 12 h after transfection but 8 h before microscopy failed to reveal any cell with cytoplasmic green fluorescence (Figure 3, G and H). Because absence of the protein synthesis inhibitor results in cytoplasmic accumulation of GFP-ER in the presence of ICI 182780 (Figure 3, A and B), continued protein synthesis appears to be required for ICI 182780-induced cytoplasmic accumulation of GFP-ER.

Confocal Laser Scanning Microscopic Examination of GFP-ER Nuclear Distribution

The ability of ICI 182780 to affect the cytoplasm–nucleus partitioning of GFP-ER suggests that other ER ligands might affect the localization of the receptor. In particular, although no change in the nuclear versus cytoplasmic compartmentalization of GFP-ER was observed for cells treated with either 17β-estradiol or 4-hydroxytamoxifen (Figure 2, compare B with D and F), the localization of GFP-ER might have been altered within the nucleus. To determine whether changes occurred in the nuclear localization of GFP-ER upon the addition of ligand, the GFP-ER-expressing MCF-7 cells were examined by high-resolution fluorescent microscopy using a confocal laser scanning microscope.

Figure 4A shows an optical section of four live MCF7 cells grown in the absence of ligand, obtained through a confocal laser scanning microscope. Note the increased resolution of the images (also other images in Figures 4 and 5) over the conventional epifluorescent images in Figures 2 and 3. Despite differences in the overall level of brightness attributable to cell-to-cell variation in the level of GFP-ER expression (e.g., Figure 4A, top left corner compared with the adjacent nucleus or the two rightmost pair of nuclei), the tagged receptor is found to be present in a reticular pattern evenly distributed throughout the nucleus. It is, however, excluded from the nucleoli. In contrast, treatment with 10 nM 17β-estradiol, 4-hydroxytamoxifen, or ICI 182780 for 1 h leads to the redistribution of the receptor, resulting in the nucleus appearing punctate and highly structured (Figure 4, B–D). Note that the ICI 182780-treated cells (1 h) failed to show cytoplasmic GFP-ER, because at this time point, few cells accumulate GFP-ER in the cytoplasm (Figures 4D and 5, D, H, and L; our unpublished results). This result is in contrast to the cytoplasmic accumulation seen after an overnight treatment (Figure 2H). Thus, intranuclear localization of the ER is altered by treatment with ER ligands (Figure 4, B–D).

Representation of Confocal Laser Scanning Microscopic Images

Because the confocal laser scanning microscope records images in a digital format, these images can be analyzed by a number of procedures. Figure 4, E and F, shows three-dimensional color surfaces in which height and color are used to represent the light intensity in the panel corresponding to the untreated or 17β-estradiol–treated cells (Figure 4, A and B). For example, the brightest nucleus in the untreated sample and the two brightest in the 17β-estradiol–treated sample are represented by the tallest nuclei whose surface color either approaches blue or is blue in Figure 4, E and F, respectively.

The fluctuation in light intensity is readily apparent when a perpendicular slice through the surface plot of the optical section for the cell is made and examined. Figure 4G, left diagram, shows a slice from an untreated nucleus (Figure 4A, green line), whereas the right diagram shows a slice from a 17β-estradiol–treated nucleus (Figure 4B, red line). In tracing along the green line from left to right in Figure 4A and examining the left diagram in the same manner for Figure 4G, it is clear that the fluorescence intensity is zero outside of the nucleus. As the line enters the nucleus, the fluorescence intensity value suddenly rises and remains fairly constant in the nucleus, except where it drops dramatically at the nucleolus, which is a little over halfway into the nucleus. The fluorescence intensity again recovers outside the nucleolus and subsequently drops to the baseline upon exiting the nucleus. A similar trend is seen for the slice of the 17β-estradiol–treated nucleus indicated in Figure 4B (right diagram); however, in this case, dramatic changes in the fluorescence intensity values are seen for the nuclear region excluding the nucleolus. Such changes mirror the punctation observed for the 17β-estradiol–treated nuclei (Figure 4B); in contrast, minor fluctuation in fluorescence intensity values in the slice of the untreated nucleus (Figure 4B, left diagram) reflects the more even, reticular distribution of GFP-ER observed in Figure 4A.

Quantitative Analysis of the Effect of Ligand on GFP-ER Nuclear Distribution

To quantitatively compare the magnitude of fluctuation in fluorescence intensity, the coefficient of variation was determined from the mean and SD of the fluorescence intensity for a segment of each line traversing the nucleus (Figure 4G, portion under the two black bars). In the case of the untreated sample, the mean fluorescence

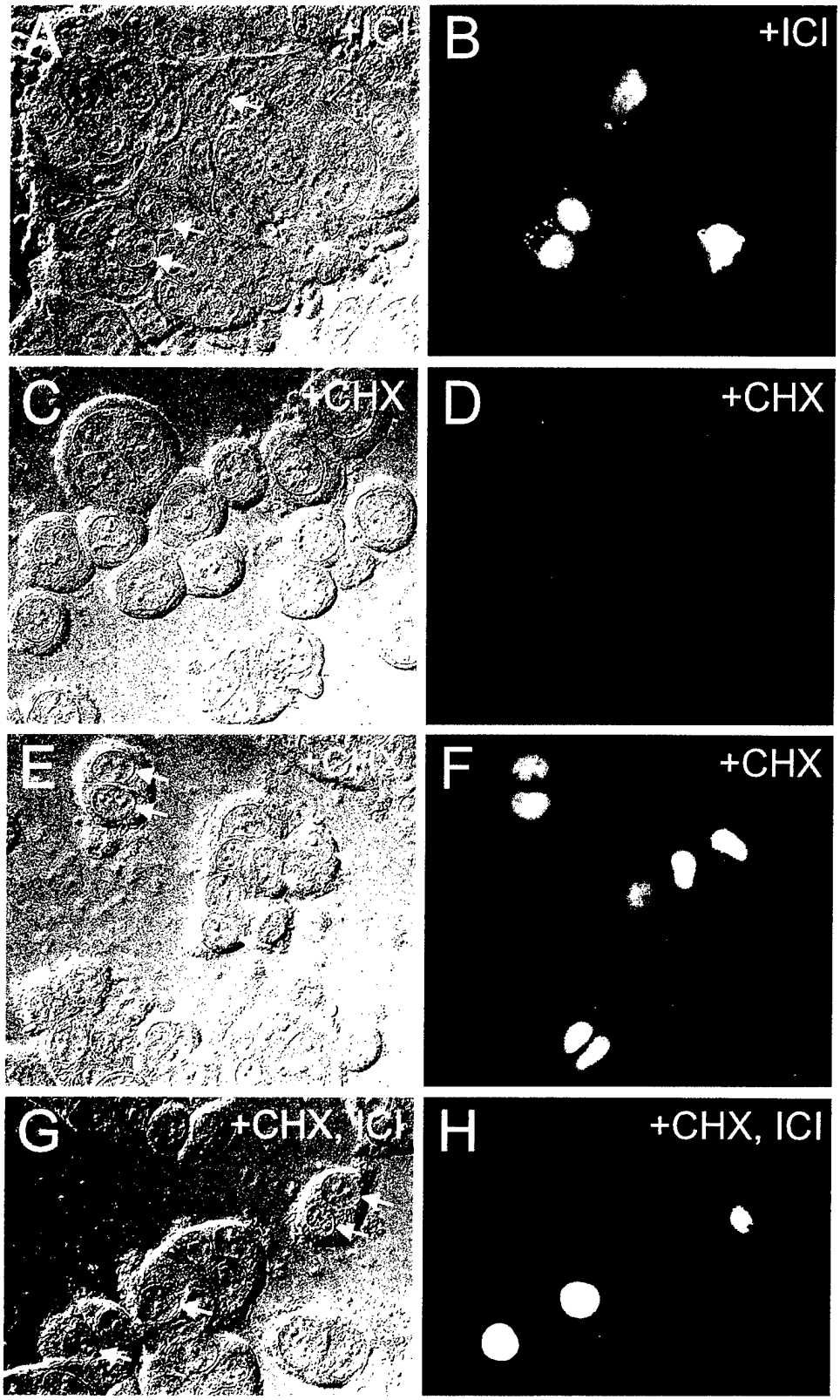

Figure 3.

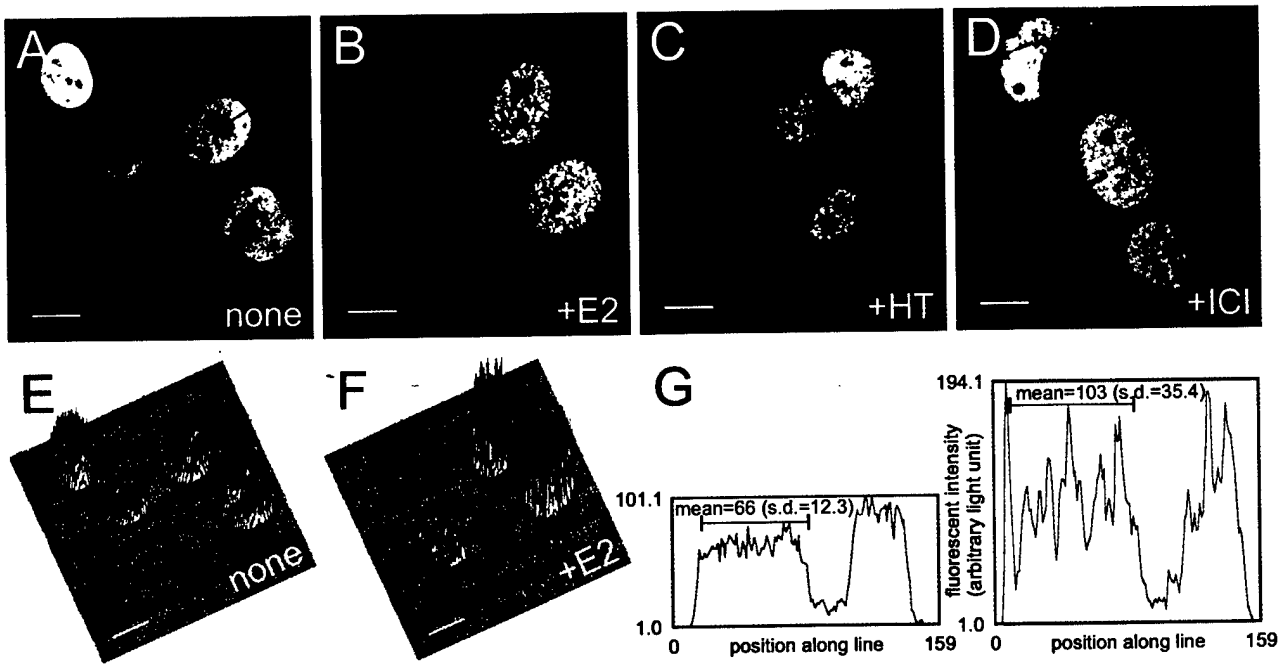

Figure 4. Effect of ligand treatment on the intranuclear distribution of GFP-ER in MCF-7 cells. MCF-7 cells were electroporated with 0.2 μg of GFP-ER expression plasmid pCI-nGL1-HEGO and cultured on coverslips overnight. The next day, media were changed, and the cells were treated with nothing (A), 10 nM 17β-estradiol (B), 10 nM 4-hydroxytamoxifen (C), or 10 nM ICI 182780 (D) for 1 h before visualization by confocal laser scanning microscopy on a Bio-Rad MRC 1024 system. Representation of fluorescence intensity in A and B is as three-dimensional plots in E and F, respectively, with the greater the fluorescence, the higher the peaks, and the cooler the colors. Bar, 10 μm. (G) Graph of fluorescence intensity along the green line for the nucleus in A (left graph with green curve) and red line for the nucleus in B (right graph with red curve). Arrowheads in A and B point to the direction of the plot from left to right in H. The black bar within each graph in H marks the segment of the line for which the mean fluorescence intensity and SDs are calculated.

intensity was 66 with an SD of 12.3, whereas the 17β-estradiol sample had a mean of 103 with an SD of 35.4. Normalization of the SD by the mean yielded the coefficient of variation, which allows direct comparison of the magnitude in the variation of the fluorescence intensity for samples with different mean values. Indeed, the coefficient of variation was nearly twofold higher for the 17β-estradiol–treated sample than for the untreated sample (0.344 vs. 0.186), indicating a substantial fluctuation in fluorescence intensity for the 17β-estradiol–treated cells than untreated cells.

To determine whether a statistically significant difference exists in the coefficient of variation after ligand treatment, the mean coefficient of variation was determined from a large population of nuclei treated in a similar manner. In the current instance, the coefficient of variation was computed in a similar manner as described in the earlier example, but now encompassing the entire nucleus in the optical section minus the nucleolus. From the coefficient of variation, a mean coefficient of variation along with its SD was calculated for each population treated in a similar manner. As seen in Table 1, the mean coefficient of variation for 164 MCF-7 cells not treated with ligand is 0.225, whereas that for 82 MCF-7 cells treated with 17β-estradiol is 0.365. Statistical Z test gives a value >26. This value indicates a chance of <1 in 10 million that these two means would show such a difference on the basis of chance alone and thereby establishes a high degree of statistical significance to these results (Chase and Bown, 1992). Similarly, treatment with 4-hydroxytamoxifen or ICI 182780 resulted in a 1.4-fold increase in the mean coefficient of variation, which is also a high statistical difference from the untreated sample.

Nuclear distribution of GFP-ER was also analyzed in another ER+ cell line, T47D, and two ER− cell lines, MDA-MB-231 and MDA-MB-435A, to determine whether the 1-h ligand treatment also caused a statis-

Figure 3 (facing page). Effect of cycloheximide treatment on ICI 182780-induced accumulation of GFP-ER in the cytoplasm. MCF-7 cells were electroporated with 0.5 μg of pCI-nGL1-HEGO DNAs and left to express for 12 h before 8 h of treatment with 10 nM ICI 182780 (A and B), 200 μg/ml cycloheximide (E and F), or 200 μg/ml cycloheximide and 10 nM ICI 182780 (G and H). Alternatively, the cells were treated with 200 μg/ml cycloheximide immediately after electroporation for 20 h (C and D). Cells were visualized by differential interference contrast (A, C, E, and G) or epifluorescence using a standard FITC filter set (B, D, F, and H). Arrows in the left panels point to nuclear fluorescence observed in the right panels.

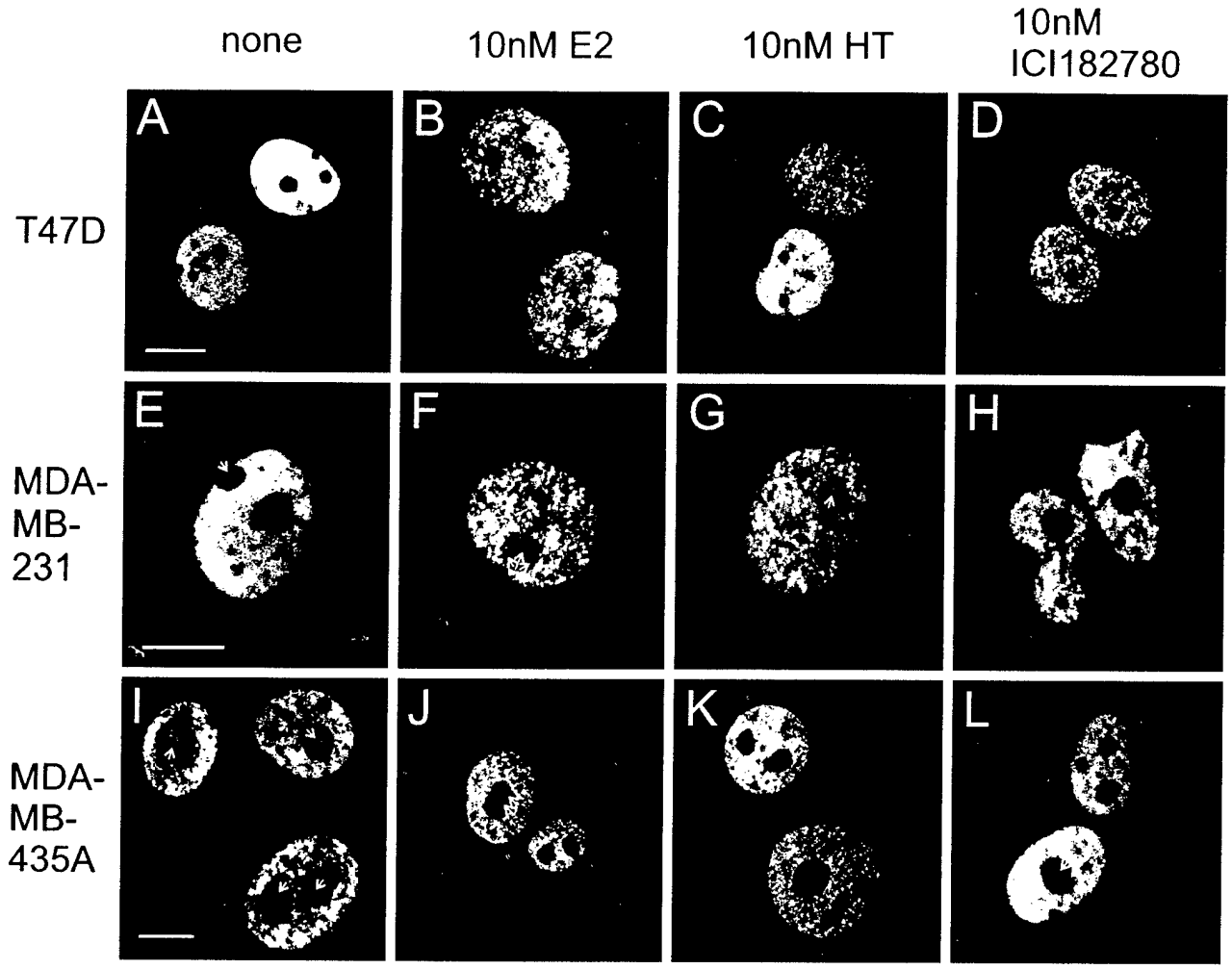

Figure 5. Effect of ligand treatment on the intranuclear distribution of GFP-ER in three human breast cancer epithelial cell lines. Three human breast cancer epithelial cell lines, T47D, MDA-MB-231, and MDA-MB-435A, were electroporated with 0.2 μg of pCI-nGL1-HEGO and treated and visualized as in Figure 3. Bar in each panel for the first column (A, E, and I), same scale bar for the other panels in the row, 10 μm. Note the presence of fluorescence in the nucleolar regions (indicated by white arrows) for the some of the MDA-MDA-231 cells (e.g., E–G), which can be quite prominent in MDA-MB-435A cells (e.g., I, J, and L).

tically significant redistribution of GFP-ER. In each case, ligand treatment results in an increase in the mean coefficient of variation (Table 1, compare leftmost number with others in each row) to a value that would occur by chance in <1 of 10 million cases. Thus, for each cell line, a significant redistribution of GFP-ER occurs upon treatment with ligand (Figure 5, compare first panel with other panels in each row).

Ligand– and Cell Line–specific Distribution of GFP-ER in the Nucleus

Although it is apparent that no two GFP-ER–expressing cells look identical (Figures 4 and 5, compare the nuclear appearance of the group of cells in each panel), each cell line has a characteristic distribution of GFP-ER and responds in a characteristic manner to ligand. For example, in the absence of ligand, GFP-ER is distributed evenly in a reticular pattern in MCF-7 cells (Figure 3A). However, deviation from this pattern occurs in T47D, MDA-MB-231, and MDA-MB-435A cells, resulting in a small but statistically significant increase in the mean coefficient of variation (Table 1, compare the value for MCF-7 with others in the first column). In the case of T47D cells, GFP-ER can be seen to concentrate at a few nuclear sites over a reticular background (more evident for the brighter nucleus in Figure 5A). The two other cell lines show an uneven distribution of GFP-ER. In the case of the MDA-MB-231 cell line, the unevenness results in the left and lower left half of the nucleus being brighter

Table 1. Mean coefficient of variation and its standard deviation obtained from the frequency distribution of nuclear fluorescence intensity for a population of cells treated in a similar manner

	None	17β-Estradiol	4-Hydroxytamoxifen	ICI 182780
MCF7	0.225 ± 0.042 (164)	0.365 ± 0.068 (82)	0.329 ± 0.059 (108)	0.326 ± 0.057 (109)
T47D	0.245 ± 0.050 (76)	0.365 ± 0.060 (60)	0.370 ± 0.084 (56)	0.373 ± 0.058 (34)
MDA-MB-231	0.245 ± 0.040 (118)	0.353 ± 0.057 (78)	0.315 ± 0.078 (54)	0.342 ± 0.074 (49)
MDA-MB-435A	0.257 ± 0.040 (84)	0.301 ± 0.040 (53)	0.287 ± 0.042 (44)	0.294 ± 0.041 (35)

Coefficient of variation is determined from the frequency distribution of the pixel intensity within each optical section of a nucleus but ignoring the nucleolus, using IPLab Spectrum software (Signal Analytics). From the coefficient of variation, the mean and its SD were determined for the number of nuclei indicated within parenthesis and are separated by the ± sign, respectively.

than the other half (Figure 5E). For MDA-MB-435A cells, the unevenness is very apparent from the "patchy" GFP-ER patterns within the nucleus (Figure 5I).

Despite the fact that GFP-ER undergoes a dramatic redistribution after ligand treatment, the magnitude of the change differs between different cell lines as each cell line responds in a characteristic manner to ligand. 17β-Estradiol treatment leads to a high degree of punctation in the distribution of GFP-ER for MCF-7 and T47D cells, less so in MDA-MB-231 cells, and least in MDA-MB-435A cells (Table 1). The extent of punctation observed for MCF-7 cells is greater for 17β-estradiol than for the antagonists, but for T47D cells, it is not significantly different. Although the GFP-ER patterns for the two antagonists are significantly different for MDA-MB-231 cells ($p <$ than 1 in 100 that the mean coefficients of variation are those in Table 1), these patterns appear not to be significantly different for MDA-MB-435A cells.

GFP-ER Presence in the Nucleolar Region

In the two ER− cell lines, expression of GFP-ER results not only in its accumulation within the nucleus but also in its presence within the nucleolar region (Figure 5, E–G, I, J, and L). This is most evident for the MDA-MB-435A cells. The extent of "nucleolar" accumulation differs between different cells in the population, independent of ligand treatment. Thus, unlike the ER+ cell lines, GFP-ER can accumulate in the nucleolar region of ER− cells.

DISCUSSION

Early attempts at localization of the ER by biochemical fractionation led to the two-step model of steroid hormone action. Binding of steroid by cytosolic steroid hormone receptor leads to its transformation and subsequent translocation to the nucleus where it regulates gene expression (Gorski *et al.*, 1968; Jensen *et al.*, 1968). This view was revised when the human ER was shown to be in the nucleus independent of ligand by immunocytology (King and Greene, 1984) and hormone-binding assays of cytoplast and nucleoplast fractions from cytochalasin B-induced enucleation of intact cells (Welshons *et al.*, 1984). Steroid hormone transformed a "loosely" bound nuclear ER to a more "tightly" bound nuclear form, which regulated gene expression (Greene and Press, 1986; Press *et al.*, 1989; Jensen, 1991). However, attempts at defining these two biochemically distinct states of ER by immunocytology failed to reveal any notable difference in the intranuclear localization of the loosely and more tightly bound forms of ER (Press *et al.*, 1985; Vazquez-Nin *et al.*, 1991; Yamashita, 1995).

In our current work, we have revisited this issue of the role of ligand in ER localization, using a direct visualization approach in living cells based on GFP tagging. We have previously shown this approach to be extremely useful for observing the subcellular localization of the GR. Using the GFP-tagging approach, we saw for the first time differences in the intranuclear distribution of the receptor that reflected the type of ligand, agonist or antagonist, bound to the receptor. That work represents the first report of the importance of ligand or signal in affecting the distribution of a steroid hormone and nuclear receptor within the nucleus (Htun *et al.*, 1996).

When ER is tagged at its amino terminus with GFP, the tagged receptor is functional by a number of criteria. First, the receptor is capable of transcriptional activation of the ERE-containing reporter gene. Second, GFP-ER responds to ligands, ER agonist or antagonist, similar to ER. Third, GFP-ER is nuclear in the absence of any added ligand, as has been reported for ER. Fourth, the pure antagonist ICI 182780 causes partial cytoplasmic accumulation of GFP-ER, as has been reported for ER (Dauvois *et al.*, 1993). Finally, GFP-ER can associate with the nuclear matrix, similar to that reported for the wild-type ER (Barrack and Coffey, 1980; Alexander *et al.*, 1987; Samuel *et al.*, 1998). Although we have not quantitatively investigated this issue, we do not see a large effect of hormone stimulation on the interaction with matrix. This could sug-

gest that the receptor is present on different matrix sites in the presence and absence of ligand.

Using this GFP-ER, we find the receptor to be distributed in a reticular pattern in the absence of ligand. This pattern suggests that the majority of the unliganded GFP-ER is not freely diffusing in the nucleus but is rather associated with some nuclear meshwork present throughout the nucleus. Ligand causes a dramatic redistribution of the receptors to numerous nuclear sites, giving a punctate nuclear pattern. These two distinct nuclear localization patterns provide the first visual evidence of the changes in receptor activity by hormone and may reflect the loose and tighter binding nuclear forms of ER (Greene and Press, 1986; Press *et al.*, 1989; Jensen, 1991). These findings also complement our earlier work with the GR, which demonstrated the importance of ligand on the nucleocytoplasmic compartmentalization and intranuclear distribution of the receptor (Htun *et al.*, 1996). Despite the fact that the current work was performed on human breast cancer epithelial cells, the nuclear redistribution of ER is expected to be a general feature of all ER-containing cells and is likely a consequence of hormone-dependent transformation of ER from a loose to tighter nuclear form.

Closer examination of the various human breast cancer epithelial cell lines shows a cell line–specific intranuclear distribution of GFP-ER. Small but significant differences in GFP-ER localization can be observed in the absence of ligand. In MCF-7 cells, GFP-ER can be seen to localize in a reticular pattern evenly distributed throughout the nucleus, excluding the nucleolus (Figure 4A). This is not so for T47D cells where a composite pattern emerged as a result of the accumulation of the receptor in a reticular pattern and to a low level of concentration at numerous nuclear sites present throughout the nucleus (Figure 5A). Despite this alteration in the nuclear pattern, comparison of different portions of the nucleus in these ER+ cells shows no remarkable difference in the fluorescent intensity and localization patterns, indicative of an even, intranuclear distribution of GFP-ER. In contrast, ER− cell lines lack this even distribution. In MDA-MB-231 cells, the receptor is slightly more abundant on one half over the other half of the nucleus (Figure 5E), whereas in MDA-MB-435A cells, the unevenness in GFP-ER distribution results in a patchy nuclear appearance (Figure 5I). Thus, each cell line shows a characteristic distribution of GFP-ER in the absence of ligand.

In comparing the confocal laser scanning microscopic images of GFP-ER-expressing cells, it is clear that no two nuclei show an identical distribution of GFP-ER despite the cells being treated and handled in a similar manner (Figures 4 and 5, compare nuclei within each panel). Differences in the phase of the cell cycle, physical characteristics, local external environment, and stochastic nature of some biological regulatory processes may contribute to a unique nuclear appearance. To describe the distribution of GFP-ER in a quantitative manner, the coefficient of variation was determined from the frequency distribution of fluorescence intensity. Although the coefficient of variation analysis ignores the precise spatial organization of GFP-ER, changes in the GFP-ER patterns are often accompanied by changes in the size and number of GFP-ER clusters and hence the frequency distribution of fluorescence intensity. Under most circumstances, the coefficient of variation will be different, and the significance of this difference can be addressed statistically. However, in cases in which the values of the coefficient of variation are similar, direct visual assessment is required to address the issue of similarity or difference in the GFP-ER patterns. Thus, the coefficient of variation serves as an indirect measure of the spatial distribution of GFP-ER through its effect on the frequency distribution of fluorescence intensity.

When cells not exposed to ER ligand are examined, a reticular pattern can be observed throughout the nuclear volume excluding the nucleolus. However, subtle differences exist (Figures 4A and 5, A, E, and I) that can be evaluated quantitatively and analyzed statistically. When the coefficient of variation was determined from the nuclear fluorescence but excluding the nucleolus, the mean coefficient of variation was smallest for MCF-7 cells, which showed an even and reticular distribution of GFP-ER (Table 1; see Figure 4A). The mean coefficient was largest for the cell line MDA-MB-435A, which deviated furthest from this distribution, as evident from the patchy appearance of the nuclei (Table 1; see Figure 5I). Intermediate values were obtained for the untreated T47D and MDA-MB-231 cells, which had a low level of deviation from the MCF-7 nuclear pattern (Table 1; see Figure 5, A and E). Heterogeneity in the observed distribution of GFP-ER is partly reflected by the SD, as indicated in Table 1. From the mean and SD, the statistical Z test established the statistical significance of the difference in the mean coefficient of variation among the different cell lines, except between T47D and MDA-MB-231 cells, and hence the existence of a unique and characteristic GFP-ER distribution pattern, at least on the average. For T47D and MDA-MB-231 cells, the mean coefficient of variation is similar; however, examination of the confocal sections has shown differences in the nuclear patterns between T47D and MDA-MB-231 cells, as discussed earlier. Thus, to a first approximation, the mean coefficient of variation for the most part adequately summarizes the different GFP-ER localization patterns and helps define a characteristic nuclear distribution of GFP-ER for each cell line.

In the presence of ligand, GFP-ER redistributes within the nucleus. Analysis of the number of cells, indicated within parentheses in Table 1, shows that for

all ligands, the greatest redistribution occurs in T47D cells, and the least occurs in MDA-MB-435A cells. The response in MCF-7 and MDA-MB-231 cells is intermediate and ligand dependent. In MCF-7 cells, 17β-estradiol caused a greater redistribution than 4-hydroxytamoxifen, which elicited a similar response as ICI 182780. However, in MDA-MB-231 cells, both 17β-estradiol and ICI 182780 caused a similar but greater change than 4-hydroxytamoxifen. Thus, each cell line not only has a characteristic GFP-ER distribution pattern but responds in a characteristic manner to ER ligands.

Responsiveness of human breast cancer to hormonal therapy correlates well with ER status in which up to 60% of ER+ tumors respond to anti-estrogen therapy, in contrast to 10% of ER− tumors (Allegra *et al.*, 1980; Samaan *et al.*, 1981; Williams *et al.*, 1987). Interestingly, treatment of ER− cells, made to express exogenous ER, with 17β-estradiol inhibited cell growth and proliferation, contrary to what is normally observed in ER+ cells (Garcia *et al.*, 1992; Jiang and Jordan, 1992; Zajchowski *et al.*, 1993; Levenson and Jordan, 1994). This differential response to the activation of ER by 17β-estradiol suggests important differences in cellular and/or nuclear content and/or structure that affects ER function. Comparing GFP-ER localization between the ER+ and ER− cell lines revealed three characteristic differences. First, GFP-ER is distributed more evenly throughout the nuclear volume minus the nucleolus in ER+ than ER− cells. Second, ER− cells had GFP-ER in the nucleolar region unlike ER+ cells, more so in MDA-MB-435A than MDA-MB-231 cells. Third, after 6–8 h of treatment with ICI 182780, GFP-ER is seen in the cytoplasm of ~90% of the ER+ cells but only 10% of ER− cells. Thus, the cell line variations in the subcellular localization of GFP-ER demonstrate that ER function is affected by cellular, nuclear, or structural differences. The importance of this alteration on the receptor's role in inhibiting the growth and proliferation of ER− cells, transfected with ER expression vector, remains to be elucidated.

Dauvois *et al.* (1993) proposed ER to constantly shuttle between the nucleus and the cytoplasm despite its predominantly nuclear location. They further reported that nuclear uptake was energy dependent and that ICI 182780 disrupted this process, resulting in the accumulation of ER in the cytoplasm. We observed that the ability of ICI 182780 to cause cytoplasmic localization of GFP-ER was prevented when breast cancer cells were incubated with both ICI 182780 and cycloheximide, a protein synthesis inhibitor. A possible explanation for this observation is that ICI 182780 prevents the nuclear uptake of newly synthesized GFP-ER. Alternatively, a labile protein factor could be required for ER to be exported from the nucleus to the cytoplasm. Variation in the abundance of this labile factor could account for the observed differences between the rate of cytoplasmic accumulation of GFP-ER in ER+ breast cancer epithelial cells and mouse ER in COS-1 cells (Dauvois *et al.*, 1993), and absence of this labile factor in 90% of the ER− cells might explain why these cells failed to show cytoplasmic green fluorescence after ICI 182780 treatment.

From our current work, it is clear that the effectiveness of ICI 182780 as an antagonist is independent of its ability to induce cytoplasmic accumulation of GFP-ER. In the ER+ human breast cancer epithelial cells, most of the GFP-ER remained in the nucleus. In an ER− human breast cancer epithelial cell, MDA-MB-435A, ICI 182780 treatment effectively suppressed GFP-ER transcriptional activation of a reporter gene; however, only 10% of these cells showed any GFP-ER accumulation in the cytoplasm. Thus, the mechanism of ICI 182780 antagonism does not depend on the nuclear–cytoplasmic recompartmentalization of the receptor but, rather, must occur within the nuclear compartment at a step required for activation of transcription by ER.

Previous studies have shown that in the absence of hormone, steroid receptors are thought not to be bound to hormone-responsive elements in target genes (Kumar and Chambon, 1988; Wijnholds *et al.*, 1988; Pham *et al.*, 1991b; McDonnell *et al.*, 1992; also reviewed in Tsai and O'Malley, 1994; Shibata *et al.*, 1997). Hormone binding causes a conformational change in the receptor that allows it to bind to its cognate sites and to regulate gene transcription. Agonist ligands induce a receptor conformation that can interact with the general transcription factors or transcriptional coactivators to establish a productive transcriptional preinitiation complex. Antagonist ligands, on the other hand, induce a different receptor conformation that interferes with the ability of the receptor to bind to DNA or alternatively to prevent formation of a productive transcriptional preinitiation complex by abrogating its interaction with the general transcription factors and/or transcriptional coactivators (Martinez and Wahli, 1989; McDonnell *et al.*, 1991; Pham *et al.*, 1991a; Sabbah *et al.*, 1991; McDonnell *et al.*, 1994, 1995; Tsai and O'Malley, 1994; Mymryk and Archer, 1995; Vegeto *et al.*, 1996; Brzozowski *et al.*, 1997; Gallo and Kaufman, 1997; Shibata *et al.*, 1997). Alternatively, antagonist ligands might promote receptor interaction with transcriptional corepressors to actively maintain a repressed transcriptional state (McDonnell *et al.*, 1992, 1994; Smith *et al.*, 1997; Lavinsky *et al.*, 1998; Zhang *et al.*, 1998). In the case of ER antagonists, antagonism appears to occur at a step subsequent to the binding of the receptor to the EREs of target genes than at the actual step of DNA binding (Martinez and Wahli, 1989; McDonnell *et al.*, 1991, 1992, 1994, 1995; Pham *et al.*, 1991a; Sabbah *et al.*, 1991; Vegeto *et al.*, 1996; Brzozowski *et al.*, 1997; Gallo and Kaufman, 1997; Shibata *et al.*, 1997). Our visual data favor a model of 4-hydroxytamoxifen and ICI 182780

antagonism in which ER binds to the hormone response elements of target genes, but its ability to activate transcription is partially or completely abolished, respectively. In support of this assertion, we find that, qualitatively, the punctate pattern observed after 4-hydroxytamoxifen or ICI 182780 treatment is no different from the pattern observed after 17β-estradiol treatment in the four human breast cancer epithelial cell lines. Quantitatively, in the case of T47D cells, no significant difference in the mean coefficient of variation can be established after 4-hydroxytamoxifen, ICI 182780, or 17β-estradiol treatment (Table 1), suggesting a similarity in the number of nuclear sites to which the ligand-bound receptor accumulates. Although we favor these sites of GFP-ER concentration as regions high in the concentration of EREs, we cannot rule out the possibility that these sites might be ER-processing sites, storage sites, or sites of interaction with the nuclear matrix.

Last, during mouse embryonic development, ER has been reported to preferentially accumulate within the nucleolar region of a specific subset of cells (Hou *et al.*, 1996). Because this recompartmentalization is observed during the ontogeny of ER− from ER+ cells, it has been suggested that this recompartmentalization might be a consequence of the mechanism involved in ER down-regulation. In this regard, we have found ER− human breast cancer epithelial cells to show GFP-ER not only in the nucleus but also in the nucleolar region (Figures 4 and 5). The presence of GFP-ER in the nucleolar region of ER− human breast cancer epithelial cells further suggests the importance of ER localization for its function and points to parallel regulatory mechanisms governing ER localization during both early mouse embryonic development and human mammary gland development.

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Chromatin, nuclear matrix and the cytoskeleton: Role of cell structure in neoplastic transformation (Review)

LAUREL T. HOLTH[1], DEBORAH N. CHADEE[1], VIRGINIA A. SPENCER[1], SHANTI K. SAMUEL[1], JANICE R. SAFNECK[2] and JAMES R. DAVIE[1]

[1]Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba R3E 0W3, [2]Department of Pathology, Health Sciences Centre and University of Manitoba, Winnipeg, Manitoba R3A 1R9, Canada

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Abstract. Aberrant nuclear and cellular structures are hallmarks of malignant transformation. Thus it is not surprising that the three-dimensional structure of the cell both affects and is affected by changes in gene expression. Here we review the role of the cytoskeleton, nuclear matrix, and chromatin structure in the genesis of cancer. The shape of a cell is governed by a dynamic tissue matrix, which includes extracellular matrix, cytoskeleton and nuclear matrix. Mechanical and chemical signals are transmitted to the nucleus, resulting in alterations in the three-dimensional chromatin organization of genes. The signal transduction pathways affect histone modifications, such as acetylation and phosphorylation, resulting in a relaxed chromatin structure observed in oncogene-transformed cells.

Contents

Correspondence to: Dr James R. Davie, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba R3E OW3, Canada

Abbreviations: AF, activating function; ER, estrogen receptor; ERE, estrogen responsive element; E_2, estradiol; HAT, histone acetyltransferase, HDAC, histone deacetylase; MAR, matrix attachment region; NMBCs, nuclear matrix proteins in breast cancer; N-CoR, nuclear receptor corepressor; Rb, retinoblastoma protein

Key words: cytoskeleton, intermediate filaments, nuclear matrix, chromatin structure, histone acetylation and phosphorylation, breast cancer, oncogene-transformed fibroblasts

1. Introduction

Pathologists have long used aberrant appearing nuclei as a diagnostic marker for cancer. Cellular transformation is accompanied by alterations in both nuclear and cytoplasmic organizations (1-6). Although extensive evidence exists based on transformation parameters such as viral and oncogenic products, much less is known regarding changes occurring in the nuclear structure that relate to the transformed phenotype. The shape of a cell is governed by a dynamic tissue matrix system that links together the three-dimensional skeletal networks from the nuclear matrix, cytoskeleton and extracellular matrix (7,8) (Fig. 1). The nuclear matrix binds to DNA regions along the chromatin fiber referred to as matrix attachment regions (MARs) (9), and, therefore, organises DNA into loop domains (10) (Fig. 2). Thus, the tissue matrix system forms a structural and functional connection between the cell periphery and the DNA, stabilizing nuclear form and integrating cell and nuclear structure (8,11,12).

Changes in the shape of the nucleus and the cytoskeleton most likely alter chromatin structure (13) and perturb the nuclear matrix (14). Changes in nuclear shape contribute to alterations in DNA synthesis and gene expression (15,16). The ß-casein gene is an excellent example of the importance of cell shape and structure in gene regulation (17). The extracellular matrix and prolactin activate the BCE-1 enhancer of this gene through at least three transcription factors binding to the enhancer and, perhaps, by altering the acetylation state of the histones. The chromatin context of the enhancer is critical because BCE-1 on a non-integrated template will not respond to extracellular and prolactin signals (18). The authors proposed two mechanisms by which the extracellular matrix induces the expression of the ß-casein gene. Changes in the three-dimensional architecture of the cell by the extracellular matrix could alter the three-dimensional structure of the nucleus and the structure and/or composition of the nuclear matrix. Perturbation in the nuclear matrix could reposition nuclear matrix-associated histone acetyltransferases (HATs) and/or histone deacetylases (HDACs) (19), resulting in the

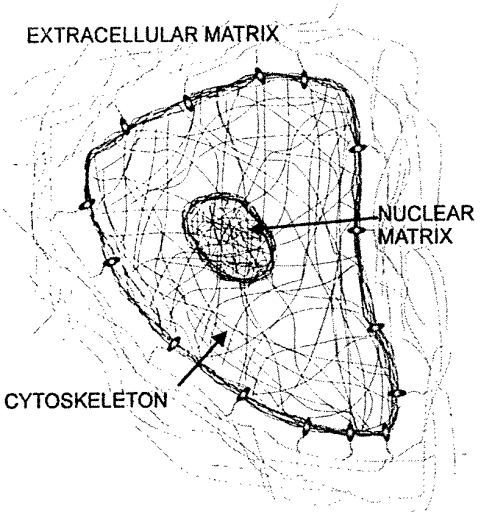

Figure 1. A model of the tissue matrix system. The tissue matrix consists of the extracellular matrix, cytoskeleton and nuclear matrix. It is important to note that the three fiber systems are interconnected and are dynamic structures.

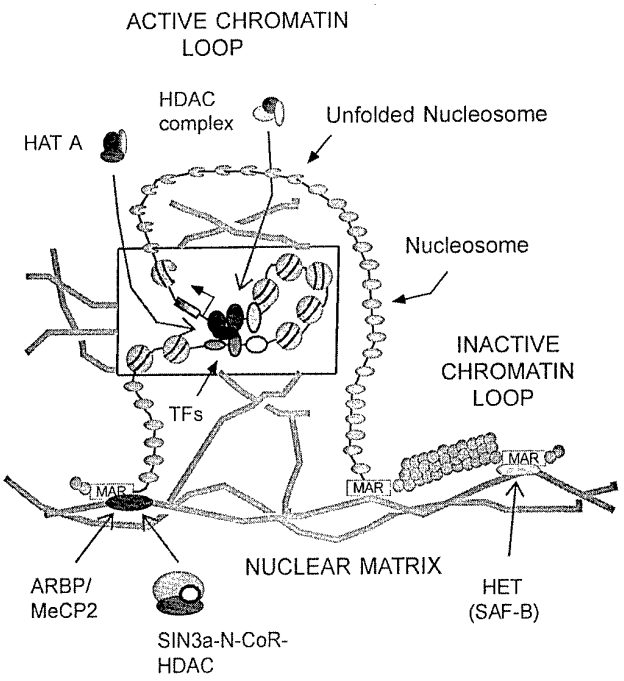

Figure 2. Organization of nuclear DNA. The inset is meant to show the multiple dynamic interactions between the nuclear matrix and regulatory and transcribed DNA sequences. TFs are transcription factors, some of which are associated with the nuclear matrix. HET/SAF-B is a MAR-binding protein that also acts as a repressor of the hsp27 gene. ARBP/MeCP2 is also a MAR binding protein that is a repressor. The nuclear matrix associated ARBP/ MeCP2 is shown recruiting the HDAC1 complex to a MAR.

remodelling by histone acetylation and transcriptional activation of the ß-casein chromatin template. Alternatively, the extracellular matrix could induce or modify cofactors, which have HDAC or HAT activity (20). Thus it can be seen from this example how the structure of a cell can play a role in the regulation of gene expression.

Cellular transformation is a result of altered gene expression, which in turn causes the expression of other genes to be altered. Here we look at the role of cell structure on the aberrant regulation of genes and in cellular transformation. Tumorigenesis involves a series of poorly understood morphological changes that lead to the development of hyperplasia, dysplasia, *in situ* carcinoma, invasive carcinoma, and in many instances finally metastatic carcinoma (21). Nuclei from different stages of disease progression exhibit changes in shape (22) and the reorganization of chromatin, which appears to correlate with malignancy (23). Thus it can be concluded that cell and nuclear structure are both affected by and affect gene expression within the cell, and therefore are likely to play a role in transformation.

2. Chromatin structure and histone modifications

Alteration in expression of specific genes involved in growth regulation can result in malignant transformation. There is an increasing awareness of the role of chromatin structure in the regulation of gene expression and in the genesis or suppression of cancer (20,24). The basic repeating unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped around a histone octamer. The histone octamer contains two each of the core histones H2A, H2B, H3 and H4 (Fig. 3). Histone H1 associates with the outer surface of the nucleosome. The H1 histones are a group of several subtypes that differ in amino acid sequence (25). H1 has a tripartite structure consisting of a central globular core and lysine rich N- and C-terminal domains. These domains interact with linker DNA to stabilize the compaction of chromatin. It has been demonstrated that both H1 and the N-terminal domain of H3 are necessary for proper chromatin folding (26-28). Due to their role in chromatin compaction, H1 histones are considered as general repressors of transcription; however, recent evidence demonstrates that H1 can function as a positive or negative gene-specific regulator of transcription (29). For example, H1b binds a regulatory element within the sequence of the replication-dependent mouse H3.2 gene, and therefore H1b may play a specific role in regulating the expression of this gene (30).

The tail domains of the core histones are involved in transcriptional regulation, replication, and chromatin condensation. The tails can undergo a context-dependent rearrangement in chromatin (31). The N-terminal domains of the core histones can be post-translationally modified by acetylation, methylation, phosphorylation and ADP-ribosylation, and the C-terminal domains of histones H2A and H2B can be ubiquitinated (10,32) (Fig. 3). Acetylated core histones are associated with transcriptionally poised and active genes (reviewed in ref. 20). The acetylation of the N-terminal tails may disrupt interactions with non-histone chromosomal proteins (33-37). Further, histone acetylation maintains the altered structure of the unfolded transcribing nucleosome (38,39) and has a role in the disruption of higher order chromatin packaging (40-43).

The chromatin fiber is organized into loops where the base of the loop (MAR) is attached to nuclear matrix proteins (10,44,45). The chromatin loop may have one or several genes. Chromatin loops containing expressed genes have a decondensed configuration that is sensitive to DNAse I

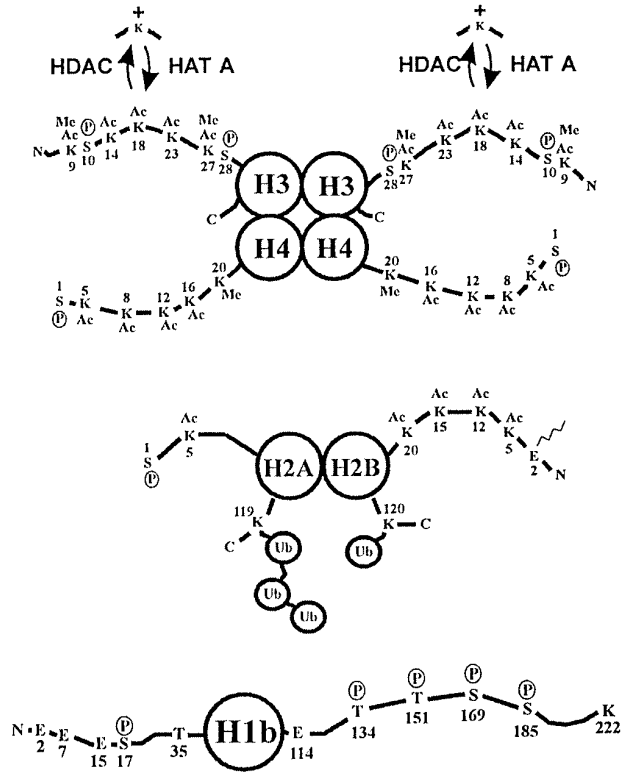

Figure 3. Post-synthetic modifications of the core and H1 histones are shown. The core histones are shown as an H2A-H2B dimer and $(H3\text{-}H4)_2$ tetramer. H3 and H4 are modified by acetylation (Ac), methylation (Me), and phosphorylation (P). H2A and H2B are modified by acetylation, phosphorylation, ubiquitination (Ub), multiubiquitination, and ADP-ribosylation (the stepladder structure). The enzymes catalyzing reversible acetylation are shown (HAT A, histone acetyltransferase; HDAC, histone deacetylase). Mouse H1b is shown in the lower part of the drawing. The potential phosphorylation sites are shown.

digestion, while loops with repressed genes have a condensed structure (Fig. 2). The boundaries of the DNAse I sensitive loop domain coincide with the position of MARs (10). These MARs delineate the loop domain in different cell types regardless of the transcriptional activity of the gene(s) within the domain. A comparison of the DNA sequences of MARs shows that they do not share extensive sequence homology; however, MAR-DNA sequences have high bending potential and may act as topological sinks (9,46-48).

Transcribing chromatin is selectively bound to the nuclear matrix (10,19,49). Multiple dynamic MARs attach transcribed chromatin regions to the nuclear matrix (see inset in Fig. 2); these MARs are different from those found at the base of loops (19). Nuclear matrix proteins, including nuclear matrix bound transcription factors (e.g., YY1 and AML), the transcription machinery, and histone modifying enzymes (e.g., HATs and HDACs) are thought to mediate the dynamic attachments between transcriptionally active chromatin and the nuclear matrix (19,49-51).

3. Role of the cytoskeleton in cancer

In eukaryotic cells, the cytoskeleton is composed of actin-containing microfilaments, tubulin-containing microtubules and intermediate filaments that are composed of keratins, desmins, and vimentin (52). The cytoskeleton is physically associated with molecules involved in chemical signalling events (11). The cytoskeleton is also associated with the nuclear matrix and may influence directly or indirectly nuclear matrix-DNA interactions, including interactions between the nuclear matrix and transcribed DNA sequences.

Intermediate filaments extend from the nucleus to the plasma membrane (53), and evidence collected from several studies suggests that intermediate filaments are enmeshed with (7,54) and penetrate the nuclear lamina (55,56). Furthermore, *in vitro* intermediate filaments bind to DNA fragments that had either MAR sequences, transcription factor motifs or structural properties important in recombination and gene expression (56). The most compelling evidence that intermediate filaments are associated with nuclear DNA *in vivo* comes from the studies of Hnilica and colleagues (55,57). They demonstrated that intermediate filaments (cytokeratins) were cross-linked to DNA *in vivo* by *cis*-diamminedichloroplatinum, an agent that preferentially cross-links nuclear matrix proteins to DNA (58). These results contradict the view that intermediate filaments are found only in the cytoplasm, and strengthen the hypothesis that intermediate filaments exist in the nucleus (56), where they may influence DNA organization and gene expression (59). It is conceivable that intermediate filaments communicate signals from the extracellular matrix to nuclear DNA, resulting in changes in gene expression. Perturbations in intermediate filament composition or structure may alter chromatin organization and lead to aberrant gene expression and the development of a malignant phenotype.

The composition of the intermediate filaments can be radically altered in cancer cells. For example, the intermediate filaments profile of breast carcinoma cells is significantly altered when compared to profiles of normal breast epithelial cells. In breast tumours, the principal cytokeratins are K8, K18 and K19 (60), whereas normal breast epithelial cells predominantly express cytokeratins K4, K5, K6, K14, and K17 (61). Likewise, the progression of rat hepatocyte nodules to a state of malignancy is accompanied by the heightened expression of cytokeratin K19 (14).

In addition to cytokeratins, the intermediate filament protein vimentin may also be involved in breast cancer development. Several studies have shown that vimentin is only expressed in breast cancer cells with a hormone independent phenotype (62,63). The overexpression of vimentin leads to the formation of aberrant vimentin structures, the co-collapse of cytokeratin K8/K18 intermediate filaments, a decrease in cytokeratin protein levels, and a distortion of nuclear shape (64). The co-expression of cytokeratins with the intermediate filament protein vimentin increases the mobility and invasiveness of human breast cancer cells (65). Thus, it is possible that vimentin is involved in the development of a metastatic phenotype in breast cancer.

The exact mechanisms responsible for altering cytoskeletal protein expression in cancer cells are unknown. Oncogenic products from the *ras* signal transduction pathway stimulate the transcription of cytokeratin 18 in a mouse embryonal carcinoma cell line (66), and cytokeratins 8 and 18 in *ras*-transformed mouse epidermal keratinocytes (67). In addition to oncogenic products, hormones regulate the expression of

intermediate filament proteins. Treatment of rat vaginal epithelium with estrogen increases the synthesis of cytokeratins (68), while androgen represses cytokeratins K8 and K18 levels in the rat ventral prostate (69). Moreover, we demonstrated that the levels of cytokeratins 8, 18 and 19 associated with the nuclear matrix were drastically reduced in an estrogen receptor (ER) positive, hormone-dependent breast cancer cell line grown in acutely estrogen-depleted conditions, while treatment of these estrogen-starved cells with estrogen restored the levels of nuclear matrix associated cytokeratin (70). This study also showed that cells chronically depleted of estrogen developed a hormone-independent phenotype and overexpressed cytokeratins K8, K18 and K19 compared to levels observed in the control cell line grown in the presence of estrogen (70).

4. Alterations in nuclear matrix composition in cancer

Important cellular processes such as DNA replication, transcription, RNA processing and transport are associated with the nuclear matrix (50,71-78). The nuclear matrix is the nuclear structure that is present following the salt extraction of nuclease-digested nuclei (79-81). It is the dynamic structural framework of the nucleus comprised of a meshwork of core filaments linked to the nuclear lamina proteins. The core filaments of the internal matrix are similar in diameter to cytoskeletal intermediate filaments (79,82,83). The nuclear matrix is thought to provide the structural support from which several nuclear processes such as DNA replication and transcription occur (84).

Several studies have shown that the protein composition of the nuclear matrix is both tissue (85) and cell type specific (86), and undergoes changes with differentiation (87-89) and transformation (86,90,91). Nuclear matrix proteins that are specific for the cell type and/or state of differentiation have been identified (92-94). These observations attest to the cell type specificity of nuclear matrix proteins.

We are interested in how the profiles of nuclear matrix proteins changed with the metastatic progression of cancerous cells. Therefore, using single oncogene transformed mouse fibroblast cell lines, we examined whether nuclear matrix protein profiles are altered in cells with increasing metastatic potential (95). Two sets of cell lines were used. The first set was derived from 10T½ fibroblasts (96). These lines were transfected with H-*ras* and the transformed foci gave rise to the cell lines CIRAS-1, -2, -3. Using criteria such as experimental metastasis assays, tumour latency, anchorage independent growth and frequency of spontaneous metastasis (97,98), it was determined that CIRAS-1 was poorly metastatic, CIRAS-2 had intermediate metastatic properties, and CIRAS-3 was highly metastatic. The second panel of cell lines was derived from NIH 3T3 fibroblasts transfected with oncogenes encoding kinases (serine/threonine or tyrosine) (99). Criteria for selection were similar to that of the 10T½ derived fibroblast cell lines. From this, it was determined that 3T3/*raf* was poorly metastatic, while 3T3/*fes* was highly metastatic. Furthermore, the highly metastatic cell lines had an altered nuclear structure (100). Using these cell lines, we found that the highly metastatic cell lines (CIRAS-3, 3T3-*fes*) had similar nuclear matrix profiles to each other that were different from the poorly metastatic cell lines (CIRAS-1, 3T3-*raf*). Clearly, this data suggests that there is a unique nuclear matrix profile for each stage of malignancy regardless of transformation agent; therefore, we decided to pursue this line of research to identify nuclear matrix biomarkers in human breast cancer.

Breast carcinoma is the most common significant cancer in women. While it is the second leading cause of cancer mortality among females, the pathogenesis of the disease remains unclear (101,102). As breast carcinoma progresses, tumour cells typically change from estrogen dependent growth to a more aggressive phenotype characterised by estrogen independent growth, resistance to endocrine therapy and a high metastatic potential (103,104). Although the most influential prognostic marker for human breast carcinoma is metastasis to the axillary lymph nodes, the presence of ERs in breast tumours is generally acknowledged as an important factor in determining the type of treatment a patient is offered (105-108). Studies have shown that both *in vivo* and *in vitro*, human breast cancer cells that lack the ER are poorly-differentiated, express vimentin, invasive, estrogen independent, and resistant to anti-estrogen therapy. Conversely, cells that express the ER are typically well differentiated, poorly metastatic, lack vimentin, and, especially at the early stages of progression, are sensitive to anti-estrogen therapy (62,108-110).

Of potential importance is the demonstration that nuclear matrix proteins can be detected in the serum and urine of cancer patients (94,111,112). Therefore it was of interest to determine if unique nuclear matrix proteins could be identified for use as diagnostic and prognostic indicators for breast cancer. In a recent study by our lab, the nuclear matrix protein profiles of a variety of human breast cancer cell lines were examined by two-dimensional gel electrophoresis (113). These human breast epithelial cancer cell lines were MCF-7, ZR-75 and T47D (ER^+/hormone dependent), MDA MB 231 and BT-20 (ER^-/hormone independent), and an ER^+/hormone independent strain of MCF-7 cells called T5-PRF. MCF-10A1, a spontaneously, immortalized human breast epithelial cell line was used as a control. Using these cell lines, we identified specific nuclear matrix proteins exclusive to ER status. These proteins were present in each of the relevant preparations (either ER^+ and/or ER^- breast cancer cell nuclear matrix proteins), but not in the preparations of nuclear matrix proteins from 'normal' breast epithelial cells. We refer to these proteins as nuclear matrix proteins in breast cancer (NMBCs), using the nomenclature proposed by Pienta and colleagues (114). Five NMBCs exclusive to ER^+ cell lines and one NMBC exclusive to the ER^- cell lines were identified (113). None of these proteins were found in MCF-10A1. As we are interested in the diagnostic and prognostic potential of these nuclear matrix proteins in human breast cancer, we looked at tumour tissue samples. Using ER^+ and ER^- human breast tumours, we were able to confirm the presence of NMBCs 1-5 in ER^+ human breast tumours and NMBC 6 in ER^- tumours (113).

Interestingly, we found that most, but not all, abundant nuclear matrix bound proteins are also bound to DNA *in situ* (44,115). This was found by *in situ* protein-DNA cross-linking by *cis*-diamminedichloroplatinum, also called cisplatin, which preferentially cross-links nuclear matrix proteins to DNA (58). Cisplatin is an antitumour drug used in the treatment of several types of cancer.

5. Transcription factors and the nuclear matrix

Transcription factors are associated with the nuclear matrix (19,49). It has been proposed that the nuclear matrix recruits transcription factors, facilitating their interaction with regulatory DNA elements (116). We modified the cisplatin cross-linking procedure to investigate whether nuclear matrix associated transcription factors were bound to nuclear matrix attached DNA *in situ* in MCF-7 cells (44). The nuclear matrix associated transcription factors and cofactors studied were: ER, a transcription factor essential to the proliferation of hormone-dependent breast cancer cells; hnRNP K, a single-strand DNA-binding transcription factor (117); HET/SAF-B, a MAR-binding protein that acts as a repressor of hsp27 gene expression (118); and HDAC1, a corepressor (44). We found that all of these nuclear matrix bound transcription factors and corepressors were cross-linked to MAR DNA *in situ* (44). In contrast, a nuclear matrix protein SRm160 involved in RNA splicing (119) was not cross-linked to DNA by cisplatin. These observations provide the first direct evidence that nuclear matrix bound transcription factors are also bound to MARs *in situ*. Further, the results provide evidence that the nuclear matrix is not simply a storage site for inactive transcription factors/cofactors. Clearly, the nuclear matrix associated transcription factors/cofactors are functional in the sense that they are bound to nuclear DNA sequences.

These results also suggest a new mechanism for the antitumour activity of cisplatin. Several mechanisms appear to be involved in cisplatin's inhibition of transcription. First, architectural factors, such as high mobility group proteins, may be 'highjacked' to cisplatin-adducted DNA (120). Second, cisplatin adducts may block transcription factor access to a regulatory DNA element (121). We suggest a third mechanism, in which the cross-linking of functionally important transcription factors/cofactors to DNA by cisplatin compromises the transcription factors/cofactors function (44). Cross-linking between the transcription factor/cofactor and MARs may interrupt their dynamic interactions and interfere with the interaction between the transcription factor or cofactor and components of the transcription machinery. The cross-linking of the corepressor, HDAC1, is of particular interest as cross-linking of this enzyme to DNA by cisplatin would effectively reduce the population of HDAC that could be recruited by transcription factors to repress specific genes [e.g., the recruitment of the Rb-HDAC1 complex by E2F (24)]. Further, HDAC is also involved in the maturation of newly synthesized chromatin (122,123). Thus, the sequestering of HDAC1 may affect transcription, cell cycle progression and replication processes.

Our finding that HDAC1 is bound to MARs *in situ* is particularly intriguing and unexpected. Previous studies showed that HDAC1 in complex with several other proteins, including the co-repressors mSin3A and N-CoR, was recruited to regulatory elements by DNA bound repressors (e.g., Mad-Max) (reviewed in ref. 20). Our results suggest that HDAC1 is associated with MARs at the base of loops. This has intriguing possibilities in chromatin architecture. Crane-Robinson and colleagues reported that at the boundaries of the DNAse I sensitive, transcriptionally competent ß-globin chromatin domain of chicken erythrocytes there is a marked change in the acetylation state of the histones (124). The ß-globin chromatin domain is associated with highly acetylated histones, while histones at the boundaries are poorly acetylated. Several studies have shown that the boundaries of a DNAse I sensitive domain co-map with MARs (10). The association of nuclear matrix bound HDAC1 with MARs would provide a mechanism by which the histones at the boundaries of the domain are deacetylated. Further support for this idea comes from two recent reports showing that MeCP2, a transcriptional repressor, recruits the HDAC complex (125,126). Strätling and colleagues found that attachment region binding protein (ARBP), a nuclear matrix associated MAR-binding protein, was MeCP2 (127). Thus, ARBP/MeCP2 anchoring MARs to the nuclear matrix could recruit the HDAC1 complex (Fig. 2). The retinoblastoma protein, a tumour suppressor, is another example of a nuclear matrix associated repressor that recruits HDAC1 (128-131). It will be interesting to know whether the recruitment of HDAC1 is a general property of nuclear matrix associated MAR-binding repressor proteins.

6. Histone modifications and transformation

There is evidence that oncogene-transformed cells have an altered chromatin structure (1,132,133). Such an alteration in chromatin structure could be a result of alterations in levels of histone subtypes or in the post-translational modification of the histones, including acetylation and phosphorylation. It has been reported that some transformed cells have alterations in the amounts of H1 variants and that this may be significant in cell transformation (134). In addition, NIH 3T3 cells transformed by the c-Ha-*ras* oncogene have a decreased level of histone H1° subtype and an increase in nucleosome repeat length (135). The authors suggest that the decrease in H1° may play a role in chromatin changes in transformed cells by weakening internucleosomal interactions and destabilizing the chromatin structure.

The analysis of the involvement of histone modifications in cellular transformation has been limited. The main exception being histone H1 phosphorylation, which has been the focus of a number of studies involving oncogene-transformed cell lines and tumour tissues of mouse. H1 C-terminal and N-terminal domains can be phosphorylated (Fig. 3). Phosphorylation of H1 has been extensively studied, and it has been proposed that H1 phosphorylation may lead to decondensation of chromatin which could facilitate access of the chromatin to factors involved in transcription and replication (136). Lennox *et al* (137) have analyzed the phosphorylated forms of H1 in cell lines derived from murine teratocarcinomas. They identified H1b as the subtype with the most highly phosphorylated isoforms. Increased phosphorylation of H1b has also been observed in tumour tissues of mouse (Lewis lung carcinoma) in comparison to normal lung tissues (137). We found an increased level of phosphorylated H1b in mouse fibroblasts transformed with oncogenes or constitutively active mitogen-activated protein (MAP) kinase (133,138). It was hypothesized that the persistent activation of the MAP kinase pathway in these cells may have altered the cyclin E associated H1 kinase activity resulting in the observed increase in phosphorylation of H1b (133) (Fig. 4). Fibroblasts lacking the tumour suppressor Rb also exhibit an increased

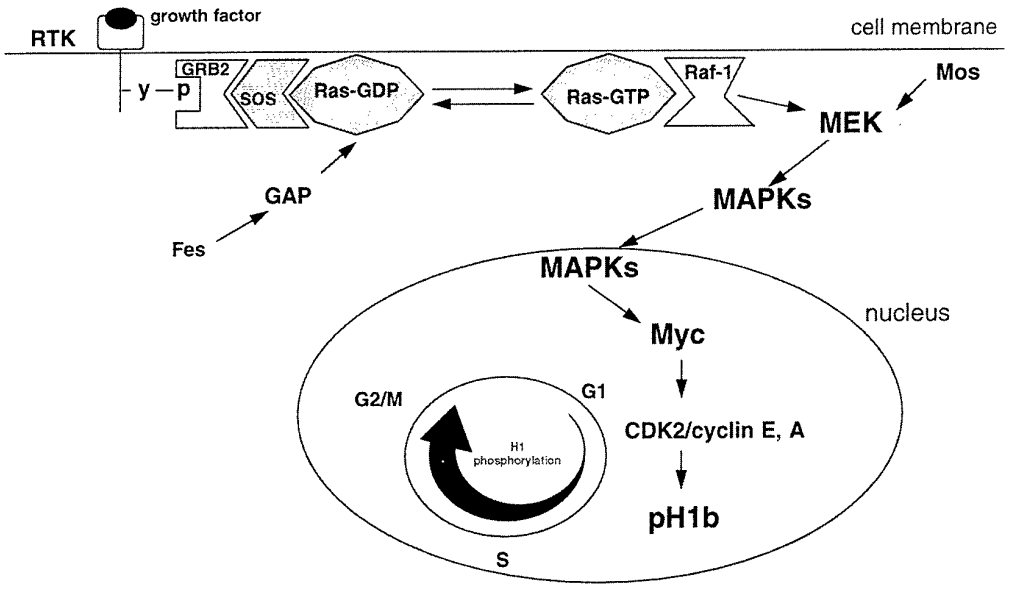

Figure 4. Histone H1b phosphorylation via the Ras-MAP kinase signal transduction pathway. In this model activated MAP kinase phosphorylates Myc, which in turn activates the cyclin E gene transcription. Cyclin E/CDK2 kinase is thought to be the H1b kinase. Increased phosphorylation of H1 throughout the cell cycle is indicated.

level of phosphorylated H1 and relaxed chromatin structure and deregulation of CDK2 may be directly involved (139). We showed that phosphorylation of H1b was dependent upon transcription and replication processes; it is the only histone modification known to be dependent upon both of these nuclear processes (140). We proposed the exposure of H1b to the cyclin E-CDK2 kinase by either the transcription or replication process results in the phosphorylation of H1b (140,141). Once modified, H1b would contribute to the relaxation of the transcribed chromatin fiber. Decondensation of the chromatin fiber would facilitate subsequent rounds of transcription.

Acetylated histones are associated with transcriptionally active and poised genes, and histone deacetylation is implicated in transcriptional repression. Until recently there had been no clear link between histone acetylation and cancer. Deregulation of the recruitment of HAT or HDAC complexes, amplification of coactivators with HAT activity, and synthesis of fusion proteins with HAT activity have been found in acute myeloid leukemia, acute promyelocytic leukemia, and breast cancer (142-149). Several groups have demonstrated that the retinoblastoma protein exists in a complex with HDAC1 and the transcriptional activator E2F (24,130,131). Thus transcriptional repression mediated by the tumour suppressor Rb, a nuclear matrix associated protein, may occur through recruitment of HDAC1. Phosphorylation of Rb by CDK4 or CDK6 results in release of Rb from E2F and the nuclear matrix (128), thus allowing transcription of E2F regulated genes, such as cyclin E. As previously mentioned, cyclin E complexed with CDK2 is thought to be involved in phosphorylation of H1 in transformed cells. Possibly, an altered chromatin structure acquired by deregulation of processes controlling histone modifications such as phosphorylation of H1 and acetylation of core histones may facilitate aberrant gene expression in the process of cellular transformation.

7. ER: a nuclear matrix associated transcription factor that affects chromatin structure

In recent years major advances have been made in our understanding of the mechanisms by which the ligand bound ER activates transcription. ER has two domains involved in transcriptional activation functions (AFs). AF-1 is located in ER's amino-terminal region, while AF-2 is located in ER's carboxyl-terminal, ligand-binding region (150). These domains interact with components of the general transcription machinery and with coactivators that have the ability to modify chromatin structure. Coactivators interacting with the estrogen bound ER (E_2-ER) include CBP/p300, SRC-1, and ACTR (also called AIB1, amplified in breast cancer 1) (for review see ref. 20). All of these coactivators are HATs. The recruited HATs would presumably acetylate neighbouring core histones (Fig. 5). Acetylation of core histones results in the relaxation of chromatin structure, probably by interfering with fiber-fiber interactions (20,42). Thus, the E_2-ER can interact with a host of proteins that may be components of the general transcription machinery (151) or remodel the chromatin template (20,150, 152), facilitating transcription and the loading of transcription factors onto regulatory DNA elements. It should be noted, however, that there are situations where E_2-ER inhibits gene expression (153).

Antiestrogens, such as hydroxytamoxifen (OH-TAM) or ICI 182,780, induce a different conformation in ER's ligand binding domain than does estradiol, resulting in the binding of a different set of cofactors (149,150,154,155). OH-TAM-ER binds to a corepressor complex, consisting of mSin3A,

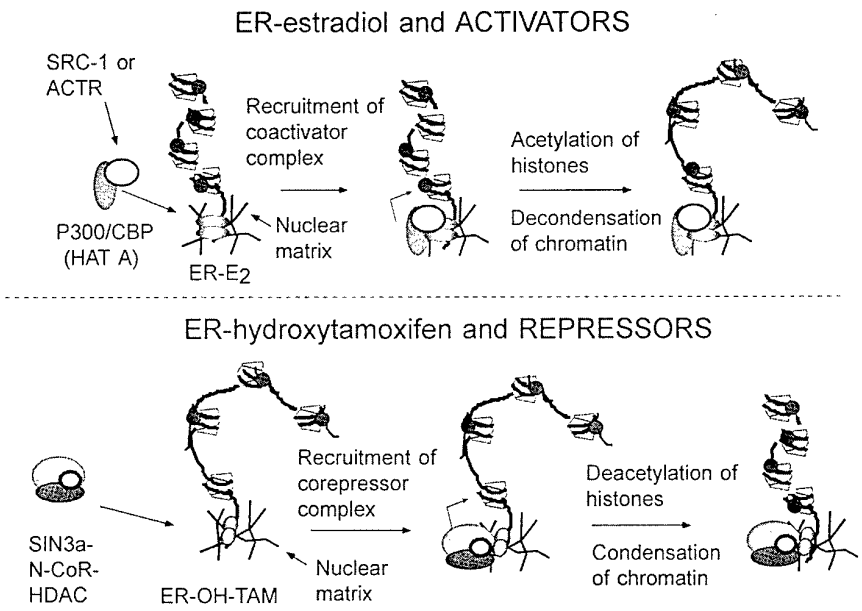

Figure 5. Model for ligand switching of ER activity. E_2-ER is shown in the top panel recruiting coactivators with HAT activity, resulting in the acetylation of neighbouring core histones and decondensation of chromatin. Below, OH-TAM is shown recruiting the HDAC complex, resulting in the deacetylation of the core histones and condensation of the chromatin fiber.

N-CoR, SAP30, HDAC1, and HDAC2 (149,156). The HDACs presumably deacetylate neighbouring core histones, resulting in the condensation of chromatin (Fig. 5). Thus, the ligands binding to ER act as molecular switches to alter the structure and function of the ER through its interaction with a battery of proteins.

To initiate these ligand-induced ER activities, the ER must be recruited to the gene's regulatory element, an estrogen response element (ERE). The classic mode of recruitment is by ER binding to a palindromic sequence through its DNA binding domain. However, ER may be recruited indirectly to regulatory DNA elements by binding to another transcription factor (e.g., Sp1) (157). Much of our knowledge of the binding of E_2-ER to a consensus ERE is from *in vitro* studies using naked DNA. From these studies, we have learned that both the ERE DNA and ER undergo a conformation change upon binding to each other (158,159). The conformational change that ER undergoes appears to depend upon the ERE sequence, and the resulting ER conformation determines which cofactors it will bind. Liganded ER can bind to the ERE as a dimer or monomer (159,160). Although we have learned much from these studies, it is important to remember that *in vivo* the regulatory DNA sequences of an estrogen responsive gene may be associated with nucleosomes. A recent study shows that E_2-ER can bind to the ERE in a nucleosome. An example is the pS2 gene, an estrogen responsive gene in breast cancer cells. Here, nucleosome positioning is key in placing the ERE in the correct position on the nucleosome such that E_2-ER can gain access to it (161).

Several studies show that ligand enhances ER binding to an ERE in chromatin (162). However, *in situ* cross-linking studies with formaldehyde suggest that unliganded ER binds to its target sites in chromatin (163). Because formaldehyde causes protein-DNA and protein-protein cross-links, it is not possible to decide whether ER was binding directly or indirectly to DNA through an intermediate DNA-binding protein.

In a perusal of characterized estrogen responsive genes expressed in human breast cancer cells, it is evident that for many of these genes ER acts through the transcription factor Sp1 (157). The Sp1$(N)_x$ERE half-site DNA-binding motif is involved in the estrogen-regulated expression of several genes, including cathepsin D (164), c-*fos* (165), retinoic receptor α (166), and hsp27 (167). The estrogen dependent expression of the c-*myc* gene in hormone dependent breast cancer cells may involve a similar Sp1 mediated recruitment of ER (168,169).

As mentioned above, we recently reported that the ER binds DNA while associated with the nuclear matrix (44). Possibly, ER brings hormone-responsive genes to nuclear matrix sites. Depending on the mode of interaction of the ER with the hormone responsive element and the ligand it is bound to, ER will recruit coactivator complexes with HAT activity or corepressor complexes with HDAC activity. These histone modifying enzymes, which are associated with the nuclear matrix, will induce a remodeling of the three-dimensional structure of chromatin. Thus, while much remains to be uncovered, the ER is an example of the interconnection between transcription factors, chromatin, nuclear structures and gene regulation.

8. Summary

In this review we have presented evidence for alterations in the composition and structure of the cell at the levels of chromatin, nuclear matrix and the cytoskeleton in cancer evolution. These different levels of structural organization are all interconnected. Change in the expression of any gene is both a cause and result of a change in its three-dimensional chromatin organization. While changes in the expression of any given gene are not

likely to cause gross morphological changes in the nuclear or cellular structure, changes in cell and nuclear shape are likely to cause alterations in the three-dimensional chromatin structure of genes and therefore affect their regulation. This view is supported by the use of aberrant nuclear shape as a diagnostic marker for cancer.

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In Situ Cross-Linking by Cisplatin of Nuclear Matrix-bound Transcription Factors to Nuclear DNA of Human Breast Cancer Cells[1]

Shanti K. Samuel, Virginia A. Spencer, Lydia Bajno, Jian-Min Sun, Laurel T. Holth, Steffi Oesterreich, and James R. Davie[2]

Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E OW3 [S. K. S., V. A. S., L. B., J-M. S., L. T. H., J. R. D.]; and Department of Medicine, Division of Medical Oncology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284 [S. O.]

ABSTRACT

Cisplatin is an antitumor drug that is used to treat several types of cancers. In this study, we analyzed the proteins that were cross-linked to DNA *in situ* in MCF-7 human breast cancer cells incubated with cisplatin. We show that cisplatin cross-links nuclear matrix proteins to DNA. In immunoblotting experiments, we found that nuclear matrix-associated transcription factors and cofactors (estrogen receptor, HET/SAF-B, hnRNP K, and histone deacetylase 1) were cross-linked to nuclear DNA. These transcription factors and cofactors have essential roles in the regulation of genes involved in the proliferation of breast cancer cells and in the organization and structure of chromatin. We applied a novel protocol to demonstrate that the nuclear matrix-bound transcription factors/cofactors were cross-linked to DNA fragments attached to the nuclear matrix. These results suggest that the cross-linking of nuclear matrix-associated transcription factors and cofactors to DNA may be one of the mechanisms by which cisplatin inhibits transcription and replication processes.

INTRODUCTION

The nuclear matrix is a dynamic three-dimensional protein-RNA structure made up of the nuclear pore lamina, internal matrix, and residual nucleoli. The nuclear matrix has roles in the organization of nuclear DNA, DNA replication, transcription, and RNA processing (1).

The chromatin fiber is organized into loops where the base of the loop is attached to NMPs[3] (2). DNA sequences called MARs mediate chromatin anchorage to the nuclear matrix. The chromatin loop may have one or several genes. Chromatin loops containing expressed genes have a decondensed configuration that is sensitive to DNase I digestion, whereas loops with repressed genes have a condensed structure. The boundaries of the DNase I-sensitive loop domain coincide with the position of MARs (2). These MARs delineate the loop domain in different cell types regardless of the transcriptional activity of the gene(s) within the domain. A comparison of the DNA sequences of MARs shows that they do not share extensive sequence homology; however, MAR DNA sequences have high bending potential and may act as topological sinks (3–5). *In vitro* assays have identified several proteins that bind to MAR DNAs, *e.g.*, lamins (6), HET/SAF-B (7, 8), hnRNP-U (SAF-A; Ref. 9), attachment region-binding protein/methyl-CpG-binding protein (10), and proteins expressed in specific cell types, such as SATB1 (11) and p114 (12). These MAR-binding proteins appear to recognize a structural feature rather than a specific sequence of the MAR DNA element. Recently, it was shown that SAF-A and SATB1 are bound to DNA *in situ* (7, 13). Kowhi-Shigematsu and colleagues (13) provided the first evidence that SATB1 binds to the base of chromatin loops *in situ*.

Transcribing chromatin is selectively bound to the nuclear matrix (2, 14, 15). Transcribed chromatin regions are attached to the nuclear matrix by multiple dynamic MARs; these MARs are different from those found at the base of loops (14). NMPs, including nuclear matrix-bound transcription factors (*e.g.*, YY1 and AML), the transcription machinery, and histone-modifying enzymes (*e.g.*, histone acetyltransferases, which are coactivators, and deacetylases, which are corepressors), are thought to mediate the dynamic attachments between transcriptionally active chromatin and the nuclear matrix (1, 14–16).

Cisplatin (*cis*-diamminedichloroplatinum), an antitumor drug that forms DNA adducts, is effective in the treatment of several cancers. Cisplatin also directly cross-links protein to DNA (cross-link distance, 4 Å; Refs. 17 and 18). Recent studies suggest that cisplatin preferentially cross-links MAR DNA to NMPs *in situ* (19, 20). Cisplatin inhibits transcription and replication processes. It has been proposed that architectural proteins (*e.g.*, high-mobility group proteins) are recruited to cisplatin-adducted DNA, taking the protein away from regulatory elements requiring the protein, leading to the repression of that gene (21, 22). Alternatively, cisplatin adducts may prevent transcription factor access to regulatory DNA sites, preventing gene expression (23).

In this study, we analyzed proteins cross-linked to DNA *in situ* in MCF-7 human breast cancer cells. We show that most abundant proteins cross-linked to DNA are NMPs. In immunoblotting studies, we determined whether nuclear matrix-associated transcription modulators were cross-linked to DNA. The nuclear matrix bound transcriptional modulators studied were: hnRNP K, a single-strand DNA-binding transcription factor; ER, a transcription factor key in the propagation of hormone-dependent breast cancer cells (24); HET/SAF-B, a MAR-binding protein that represses the expression of hsp27 (8); and histone deacetylase, a corepressor associated with mSin3A, N-CoR, and other proteins that are recruited by DNA-binding proteins, including Mad-Max and hormone receptors (*e.g.*, ER-hydroxytamoxifen), to repress transcription (25). We show that these transcriptional modulators are cross-linked to DNA by cisplatin. To test if the nuclear matrix-associated transcription factors were bound to MAR DNA *in situ*, we devised a novel strategy to isolate NMPs cross-linked to MAR DNA fragments. We show that nuclear matrix-isolated transcription factors ER, HET/SAF-B, hnRNP K, and histone deacetylase are cross-linked to MAR DNA *in situ* by cisplatin.

MATERIALS AND METHODS

Cell Culture. The human breast carcinoma cell line used in this study, MCF-7, is ER^+ and hormone dependent. The cell line was maintained at 37°C (humidified atmosphere, 5% CO_2/95% air) on 245 × 245 × 25 mm tissue culture trays (Nunc/Life Technologies, Inc., Mississauga, Ontario, Canada) in culture medium containing DMEM (Life Technologies, Inc., Mississauga, Ontario) supplemented with 1% (v/v) L-glutamine, 1% (v/v) glucose, 1% (v/v)

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[2] To whom requests for reprints should be addressed, at Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E OW3. Phone: (204) 789-3215; Fax: (204) 789-3900; E-mail: davie@cc.umanitoba.ca.

[3] The abbreviations used are: NMP, nuclear matrix protein; MAR, matrix attachment region; ER, estrogen receptor; IF, intermediate filament; NM1-IF and NM2-IF, nuclear matrix with associated IF 1 and 2, respectively.

penicillin/streptomycin, and 5% (v/v) FCS (Life Technologies, Inc.). The cells were cultured and consecutively passaged at least three times. At ~90% confluence, cells were removed from the trays with a cell scraper and frozen as pellets containing 1×10^7 cells at −70°C.

To assess viability of MCF-7 cells at various concentrations of cisplatin, cells were plated at 1×10^3 in 35-mm round dishes in culture medium. Cells were allowed to attach for 4 h, and then the culture medium was replaced with fresh medium and various concentrations of cisplatin (35, 100, 500, or 1000 μM) in Hanks' buffer with NaCl substituted with an equal molarity of sodium acetate. For the control, Hanks' buffer, containing sodium acetate instead of NaCl, without cisplatin, was added. At the end of each time point, the medium was removed by aspiration, and 1 ml of Earle's-EDTA was placed in dishes to lift cells (2 min at 37°C). Once cells had lifted, the cells were counted in a Coulter counter.

Isolation of NMPs. Nuclear matrices were prepared according to a procedure that was reported previously (26), but with the dialysis step omitted. In brief, nuclei from cell pellets (1×10^7) were resuspended to a concentration of 20 A_{260}/ml and digested with DNase I (Sigma Chemical Co., St. Louis, MO) for 20 min at room temperature. Ammonium sulfate (final concentration, 0.25 M) was added, and the NM1-IF pellet was obtained by centrifugation. The NM1-IF pellet was then extracted with a buffer containing 2 M NaCl. The resulting pellet (NM2-IF) was collected by centrifugation and resuspended in appropriate volumes of 8 M urea, aliquotted, and frozen at −20°C. Alternatively, the NM2-IF nuclear matrices were solubilized, and IF proteins removed as described previously (26). Phenylmethylsulfonyl fluoride was included in all buffers. Prior to gel electrophoresis, protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad, Richmond, CA) with BSA as a standard.

Isolation of Proteins Cross-Linked to DNA *in Situ*. DNA-protein cross-linking was performed as described previously (19, 20). Briefly, MCF-7 cells at a density of 1×10^6 cells/ml were resuspended in Hank's buffer containing sodium acetate instead of NaCl at the same concentration and 35, 100, 500, or 1000 μM cisplatin. The cells were incubated at 37°C for 2 h with gentle shaking. Following this incubation, cells were treated with lysis buffer (5 M urea, 2 M guanidine-HCl, 2 M NaCl, and 0.2 M potassium phosphate, pH 7.5). Hydroxylapatite (4 g/20 A_{260} units of lysate; Bio-Rad) was then added. The hydroxylapatite resin was washed with lysis buffer to remove RNA and proteins not cross-linked to DNA. To reverse the cross-linking, the hydroxylapatite was incubated in lysis buffer containing 1 M thiourea instead of 5 M urea. By doing so, the proteins were released from hydroxylapatite, while the DNA remained bound. The released proteins were dialyzed overnight against double-distilled water and lyophilized. The lyophilized protein preparation was resuspended in 8 M urea. Phenylmethylsulfonyl fluoride was included in all buffers. Protein concentrations were determined using the Bio-Rad protein assay, as described above.

Isolation of NMPs Cross-Linked to MAR DNA *in Situ*. This novel methodology used a combination of the protocols described above. In brief, MCF-7 cells were cross-linked with cisplatin. Following cross-linking, the nuclear matrices (NM1-IF and NM2-IF) were isolated and solubilized in lysis buffer. NMPs cross-linked to the nuclear matrix-bound DNA fragments were isolated by hydroxylapatite column chromatography.

One- and Two-Dimensional PAGE. One-dimensional SDS 8% polyacrylamide and two-dimensional gel electrophoresis were performed as described previously (26).

Immunoblot Analyses. NMPs electrophoresed on SDS-polyacrylamide gels were transferred to nitrocellulose (Bio-Rad) as previously described (27). The resulting nitrocellulose filters were immunochemically stained with anti-human HDAC1 (28), anti-ER (H222; a gift from Dr. G. Greene), anti-HET (8), and anti-B1C8 (29) antibodies, respectively, and followed by either goat antirabbit, goat antimouse, or goat antirat (Bio-Rad) antibodies conjugated to horseradish peroxidase. The immunochemical staining was detected using the enhanced chemiluminescence detection system (Amersham, Mississauga, Ontario, Canada).

RESULTS AND DISCUSSION

MCF-7 breast cancer cells (ER^+ and hormone dependent) were incubated with varying concentrations of cisplatin to determine the effect of cisplatin on cell growth and viability. Cisplatin at 35 μM reduced growth of the breast cancer cells; however, at higher concentrations of cisplatin, cell growth and survival were greatly affected (Fig. 1). At 100 μM or higher concentrations of cisplatin, the cytotoxicity of cisplatin became increasingly evident.

To isolate the proteins cross-linked to DNA *in situ*, cisplatin-treated (1 mM for 2 h) MCF-7 breast cancer cells were lysed in a denaturation buffer, the DNA was bound to hydroxylapatite, and cross-links between protein and DNA were reversed with thiourea. The resulting protein preparation was analyzed by two-dimensional gel electrophoresis. To find if the proteins cross-linked to nuclear DNA by cisplatin were NMPs, proteins from the NM1-IF nuclear matrix fraction were also analyzed by two-dimensional gel electrophoresis. Fig. 2 shows that the two-dimensional gel patterns of NMPs and proteins cross-linked to DNA were similar. The NMPs lamins A and C, for example, were found in both protein preparations.

Mattern *et al.* (30) have shown that hnRNPs are abundant NMPs in HeLa cells. One of these hnRNP proteins, hnRNP K, is a single-strand DNA-binding phosphoprotein important in the transcriptional regulation of the c-*myc* gene and other genes (31). *In vitro* studies show that hnRNP K interacts with TATA-binding protein, suggesting that nuclear matrix-associated hnRNP K has the potential to recruit general transcription factors and RNA polymerase transcription machinery to the nuclear matrix (31). Fig. 2 shows that hnRNP K was associated with the nuclear matrix of MCF-7 breast cancer cells and was cross-linked to DNA by cisplatin. This finding suggests that a nuclear matrix-associated transcription factor was cross-linked to DNA by cisplatin.

In immunoblotting experiments, we investigated whether other transcription factors important to the proliferation of MCF-7 breast cancer cells were cross-linked to DNA. Fig. 3 shows that ER was associated with nuclear matrices of MCF-7 cells and that this transcription factor was cross-linked to nuclear DNA. We have shown previously that HET/SAF-B is associated with the nuclear matrix of breast cancer cells (8), and the results shown in Fig. 3 confirm this result. HET/SAF-B was originally discovered by its ability to bind MAR DNA (7); this NMP has a role in the organization of nuclear

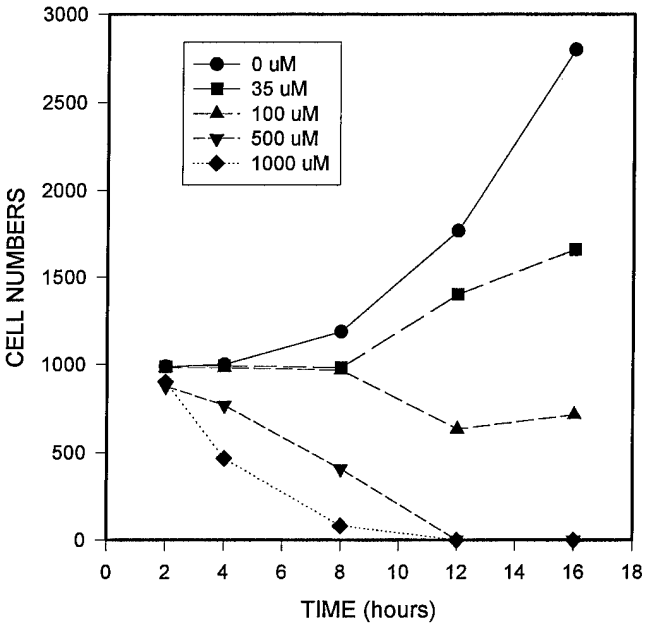

Fig. 1. Incubation of MCF-7 human breast cancer cells with cisplatin. MCF-7 breast cancer cells were plated at 1×10^3 cells and incubated with 0, 35, 100, 500, and 1000 μM cisplatin for the times indicated. The data shown are representative of results of three experiments.

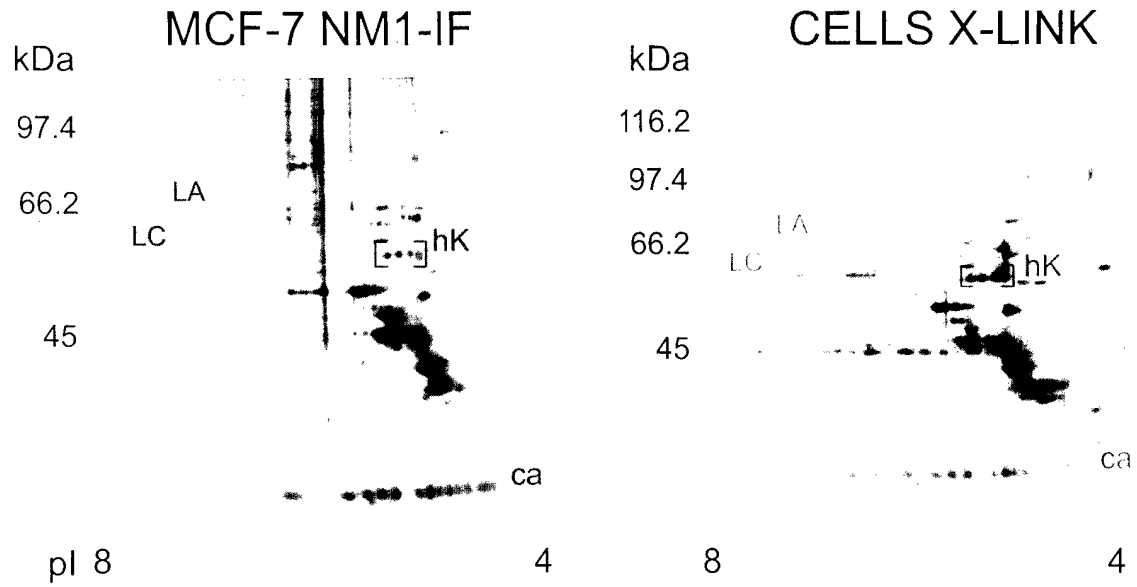

Fig. 2. NMPs cross-linked to DNA by cisplatin. Forty μg of NMPs (*MCF-7 NM1-IF*) and proteins (40 μg) cross-linked to DNA by 1 mM cisplatin (*CELLS X-LINK*) from MCF-7 human breast cancer cells were electrophoretically resolved on two-dimensional polyacrylamide gels. The gels were stained with silver. *ca*, positions of the carbamylated forms of carbonic anhydrase. The positions of the molecular weight standards (in kDa) are shown on the *left*. *LA*, lamin A; *LC*, lamin C; *hK*, hnRNP K.

DNA and in transcriptional repression (8). Fig. 3 shows that HET/SAF-B was cross-linked to nuclear DNA by cisplatin.

We had shown previously that histone deacetylase activity is associated with the avian erythrocyte and hepatocyte nuclear matrix (14). Fig. 3 shows that histone deacetylase 1 was associated with the nuclear matrix of MCF-7 breast cancer cells. Histone deacetylase 1 is a corepressor that is a component of several multiprotein complexes that may contain Sin3A-N-CoR (32, 33), YY1 (34), or Rb (35). Histone deacetylase is also found associated with transcribed chromatin, where the enzyme catalyzes dynamic histone acetylation (14). Because histone deacetylase is recruited by sequence-specific DNA-binding proteins to specific gene regulatory sites, we did not expect to find histone deacetylase associated with nuclear DNA. However, contrary to our expectations, Fig. 3 shows that histone deacetylase 1 was cross-linked to nuclear DNA by cisplatin.

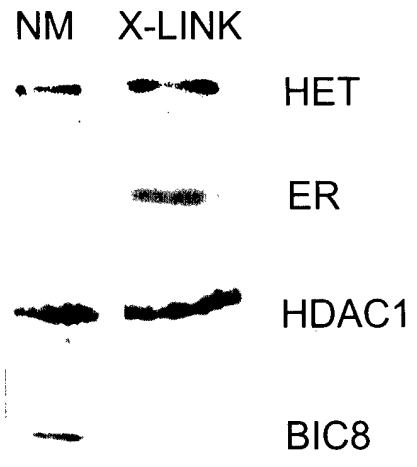

Fig. 3. Transcriptional modulators cross-linked to DNA by cisplatin. NMPs with IF proteins removed (*NM*) and proteins cross-linked to DNA of MCF-7 human breast cancer cells by 1 mM cisplatin (*X-LINK*) were electrophoretically resolved on SDS-8% polyacrylamide gels and transferred to nitrocellulose. The appropriate antibody was used to immunochemically stain the Western blot for: *HET*, HET/SAF-B; *ER*; *HDAC1*, histone deacetylase-1; and *B1C8*, SRm160. Twenty μg of protein were used for all immunoblots except for the detection of *HET*, for which 40 μg of protein were used.

The above studies show that four nuclear matrix-bound transcription factors, hnRNP K, ER, HET/SAF-B, and histone deacetylase, were cross-linked to nuclear DNA by cisplatin. To test for the specificity of the cross-linking of proteins to DNA, we examined the protein preparations containing NMPs and proteins cross-linked to DNA for the presence of B1C8. B1C8 (more recently called SRm160) is a NMP that functions as a coactivator of pre-mRNA splicing and would not be expected to be associated with DNA (36). Fig. 3 shows that B1C8 was associated with the nuclear matrix of breast cancer cells but that it was not cross-linked to DNA by cisplatin.

The cross-linking of transcription modulators ER, HET/SAF-B, and histone deacetylase to nuclear DNA as a function of cisplatin concentration was studied. Fig. 4 shows that the pattern of abundant proteins cross-linked to DNA by 35, 100, 500, and 1000 μM cisplatin following a 2-h incubation did not change appreciably. However, immunoblot analysis revealed that cross-linking of ER and HET-SAF-B was more efficient at 500 and 1000 μM cisplatin than it was at 35 and 100 μM. In contrast to ER and HET-SAF-B, the cross-linking of histone deacetylase 1 to DNA appeared to be equally efficient at all concentrations of cisplatin used.

Because NMPs appear to be preferentially cross-linked to DNA by cisplatin, we postulated that the nuclear matrix-associated transcriptional modulators hnRNP K, ER, HET-SAF-B, and histone deacetylase were cross-linked to MAR DNA. We tested this idea by designing a novel protocol in which MCF-7 cells were first incubated with cisplatin, followed by the isolation of nuclear matrix fractions NM1-IF or NM2-IF. The DNA remaining associated with these fractions, which was typically 2–3% and <1% of nuclear DNA for NM1-IF and NM2-IF, respectively, was added to hydroxylapatite. Following washes of the hydroxylapatite slurry, cross-links between the proteins and nuclear matrix-associated DNA (*i.e.*, MAR DNA) were reversed with thiourea. The proteins cross-linked to MAR DNA of NM1-IF and NM2-IF were analyzed by two-dimensional gel electrophoresis (data not shown) and by immunoblotting. Analysis of the two-dimensional gel patterns of the proteins cross-linked to nuclear

matrix attached DNA showed that hnRNP K was cross-linked to MAR DNA from NM1-IF and NM2-IF fractions (data not shown). Fig. 5 shows that the transcription modulators ER, HET/SAF-B, and histone deacetylase 1 were cross-linked to MAR DNA *in situ.*

These observations provide the first direct evidence that nuclear matrix-associated transcription factors/cofactors are bound to MAR DNA *in situ.* Furthermore, the results provide evidence that the nuclear matrix is not simply a storage site for inactive transcription factors/cofactors. Clearly, the nuclear matrix associated transcription factors/cofactors are functional, in the sense that they are bound to nuclear DNA sequences.

Several mechanisms appear to be involved in cisplatin's inhibition of transcription. First, architectural factors, such as high-mobility group proteins, may be "hijacked" to cisplatin-adducted DNA (22). Second, cisplatin adducts may block transcription factor access to a regulatory DNA element (23). We suggest a third mechanism, in which the cross-linking of functionally important transcription factors/cofactors to DNA by cisplatin compromises the transcription factors/cofactors function. Cross-linking between transcription factors/cofactors and DNA was observed after 2 h at 35 μM cisplatin, and it has been reported that cisplatin protein-DNA cross-links are repaired slowly (17). The interactions between nuclear matrix-bound transcription factors and transcribed DNA is thought to be dynamic. Cross-linking between the transcription factor/cofactor and MAR DNA may not only interrupt this dynamic process but may also interfere with the interaction between the transcription factor or cofactor and components of the transcription machinery. The cross-linking of the corepressor histone deacetylase 1 is of particular interest because cross-linking of this enzyme to DNA by cisplatin would effectively reduce the population of histone deacetylase that could be recruited by transcription factors to repress specific genes [*e.g.*, the recruitment of the Rb-HDAC1 complex by E2F (35)]. Furthermore, histone deacetylase is also involved in the maturation of newly synthesized chromatin (37, 38). Thus, the sequestering of histone deacetylase may affect transcription, cell cycle progression, and replication processes.

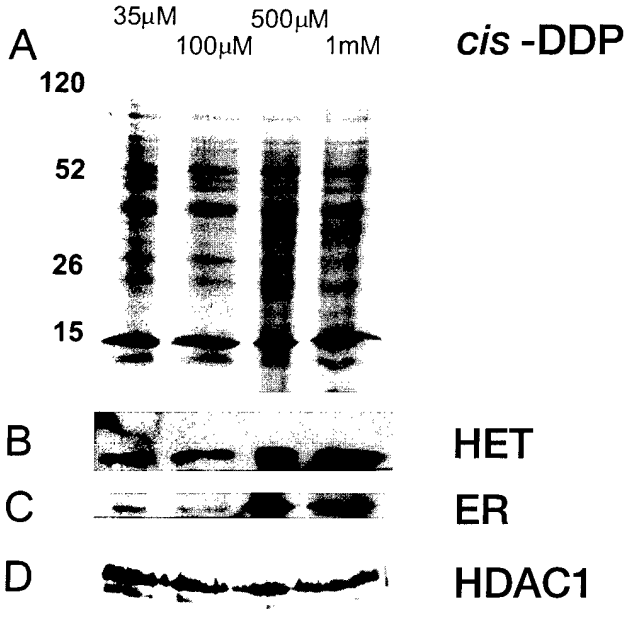

Fig. 4. Cross-linking of transcriptional modulators to DNA by different concentrations of cisplatin. MCF-7 breast cancer cells were incubated with 35, 100, 500, or 1000 μM cisplatin for 2 h, and proteins that were cross-linked to DNA were isolated. For each preparation, the yield of protein was 160 μg from 1×10^7 cells. *A*, proteins (20 μg) that were cross-linked to DNA were electrophoretically resolved on SDS-8% polyacrylamide gels, which were stained with Coommassie blue. The positions of the molecular weight standards (in thousands) are shown on the *left.* The proteins were transferred to nitrocellulose, and *HET* (HET/SAF-B; *B*, 40 μg), *ER* (*C*, 20 μg), and *HDAC1* (histone deacetylase 1; *D*, 20 μg) were detected by immunochemical staining with the appropriate antibody.

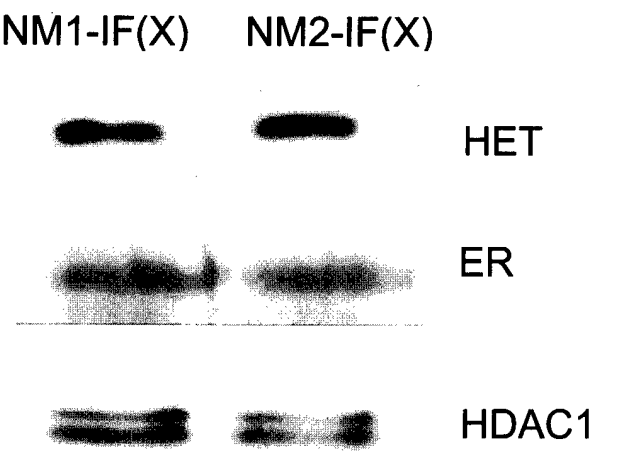

Fig. 5. Cisplatin cross-linking of transcriptional modulators to MAR DNA. Nuclear matrices NM1-IF and NM2-IF were isolated from MCF-7 breast cancer cells incubated with 1 mM cisplatin for 2 h [*NM1-IF(X)* and *NM2-IF(X)*, respectively]. The proteins (20 μg, except for *HET*, for which 40 μg of protein were loaded) cross-linked to MAR DNA were electrophoretically resolved on SDS-8% polyacrylamide gels. *HET* (HET/SAF-B), *ER*, and *HDAC1* (histone deacetylase 1) were detected on Western blots with the appropriate antibody.

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Histone Acetylation Is Required to Maintain the Unfolded Nucleosome Structure Associated with Transcribing DNA*

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Harminder Walia, Hou Yu Chen, Jian-Min Sun, Laurel T. Holth, and James R. Davie‡

From the Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba R3E OW3, Canada

Nucleosomes associated with transcribing chromatin of mammalian cells have an unfolded structure in which the normally buried cysteinyl-thiol group of histone H3 is exposed. In this study we analyzed transcriptionally active/competent DNA-enriched chromatin fractions from chicken mature and immature erythrocytes for the presence of thiol-reactive nucleosomes using organomercury-agarose column chromatography and hydroxylapatite dissociation chromatography of chromatin fractions labeled with [^{3}H]iodoacetate. In mature and immature erythrocytes, the active DNA-enriched chromatin fractions are associated with histones that are rapidly highly acetylated and rapidly deacetylated. When histone deacetylation was prevented by incubating cells with histone deacetylase inhibitors, sodium butyrate or trichostatin A, thiol-reactive H3 of unfolded nucleosomes was detected in the soluble chromatin and nuclear skeleton-associated chromatin of immature, but not mature, erythrocytes. We did not find thiol-reactive nucleosomes in active DNA-enriched chromatin fractions of untreated immature erythrocytes that had low levels of highly acetylated histones H3 and H4 or in chromatin of immature cells incubated with inhibitors of transcription elongation. This study shows that transcription elongation is required to form, and histone acetylation is needed to maintain, the unfolded structure of transcribing nucleosomes.

Acetylation of the core histones (H2A, H2B, H3, and H4) is a dynamic process catalyzed by histone acetyltransferases and histone deacetylases (1, 2). In chicken immature erythrocytes, 4% of the modifiable lysine sites participate in dynamic histone acetylation. These core histones are rapidly acetylated ($t_{1/2}$ = 12 min for monoacetylated H4) and rapidly deacetylated ($t_{1/2}$ = 5 min for the tetraacetylated isoform of H4) (3, 4). Histones undergoing rapid acetylation and deacetylation are associated with transcriptionally active chromatin (5–7). The recent findings that histone acetyltransferases and deacetylases are transcriptional coactivators and corepressors have increased our understanding of how the process of dynamic histone acetylation is established on transcriptionally active chromatin (2, 8).

Transcriptionally active chromatin has a soluble and insoluble nature (9). Transcribed DNA is found in chromatin fragments that are soluble in 0.15 M NaCl and/or 2 mM $MgCl_2$ and in chromatin fragments associated with the low salt-insoluble residual nuclear material (nuclear skeletons) (for review, see Davie (10)). Chromatin engaged in transcription is thought to be retained by the nuclear skeleton by multiple dynamic attachments between the nuclear matrix and transcribed chromatin; hence rendering the transcribing chromatin insoluble (11, 12). As histone acetyltransferase and deacetylase activities are associated with the nuclear matrix (7, 13), we proposed that these nuclear matrix-bound enzymes may mediate some of the dynamic attachments between active chromatin and nuclear matrix (13, 14).

Most information on the structure and composition of transcriptionally active nucleosomes is from studies that analyze soluble transcriptionally active chromatin. However, most of the transcribed chromatin fragments partition with the low salt-insoluble nuclear material (nuclear skeleton) (7, 15, 16). We presented evidence that dynamically acetylated histones are associated with the nuclear matrix-bound transcriptionally active chromatin (7). Otherwise, little is known about the structure and composition of transcribing nucleosomes attached to the nuclear skeleton.

Allfrey and co-workers demonstrated that nucleosomes in the transcribed regions of soluble chromatin of mammalian cells unfold exposing the cysteinyl-thiol groups of histone H3 (17, 18). The unfolding of the nucleosome was dependent upon ongoing transcription. Exploiting this feature of transcribing nucleosomes, a procedure to isolate soluble transcriptionally active chromatin by organomercury-agarose affinity chromatography was developed. The transcribing chromatin was associated with highly acetylated histones (18–20). However, current evidence argues that histone acetylation is not involved in the generation of the unfolded nucleosome. Reconstitution of nucleosomes with highly acetylated histones did not result in the formation of a thiol-reactive nucleosome (21). Further, treating mammalian cells with the histone deacetylase inhibitor, sodium butyrate, did not increase the level of thiol-reactive nucleosomes (6).

Analysis of chicken mature erythrocyte salt-soluble polynucleosomes highly enriched in transcriptionally competent DNA and highly acetylated histones (22, 23) showed that this chromatin fraction lacked thiol-reactive nucleosomes.[1] To address the question of whether unfolded nucleosomes exist in chicken erythrocytes, we investigated the H3 thiol reactivity of salt-soluble and low salt-insoluble (nuclear skeleton-associated) chromatin from mature (transcriptionally silent) and immature (transcriptionally active) chicken erythrocytes. We report that the thiol-reactive, unfolded nucleosome exists in imma-

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‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Ave., Winnipeg, Manitoba R3E 0W3, Canada. Tel.: 204-789-3215; Fax: 204-789-3900; E-mail: Davie@cc.umanitoba.ca.

[1] J. A. Ridsdale, P. Fredette, and J. R. Davie, unpublished observations.

ture, but not mature, erythrocyte salt-soluble chromatin fragments and chromatin fragments associated with the nuclear skeleton. However, histone deacetylase activity had to be suppressed to detect thiol-reactive nucleosomes in immature erythrocyte chromatin. These studies show that highly acetylated histones maintain the unfolded nucleosome structure formed by transcriptional elongation.

EXPERIMENTAL PROCEDURES

Isolation and Treatments of Immature and Mature Chicken Erythrocytes—Mature and immature erythrocytes were isolated from normal and anemic young adult White Leghorn chickens, respectively, as described previously (24). Immature and mature erythrocytes were collected in 75 mM NaCl and 25 mM EDTA, while cells to be incubated with sodium butyrate or trichostatin A were collected in 75 mM NaCl, 25 mM EDTA, and 30 mM sodium butyrate. Cells were incubated at 37 °C in Swims S-77 medium (Sigma) with 30 mM sodium butyrate or 100 ng/ml trichostatin A for 90 min to prevent the deacetylation of highly acetylated histones. To inhibit transcription elongation, cells were incubated at 37 °C for 90 min with transcription inhibitors actinomycin D (0.04 μg/ml) (24), 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)[2] (75 μM) (24, 25), or camptothecin (20 μM) (26) followed by a 90-min incubation with or without 30 mM sodium butyrate.

Erythrocyte Chromatin Fractionation—The fractionation of chromatin was done as described previously (24). All buffers contained 1 mM phenylmethylsulfonyl fluoride. Briefly, nuclei (50 A_{260} units/ml) were digested with micrococcal nuclease (15 A_{260} units/ml for 25 min at 37 °C), collected by centrifugation, and then resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Following centrifugation the soluble chromatin fraction (fraction SE) and low salt insoluble chromatin fraction (nuclear skeleton, PE fraction) were isolated. The chromatin fragments of fraction SE were further fractionated by the addition of NaCl to 150 mM. Following centrifugation, chromatin fractions P150 (pellet) and S150 (salt-soluble chromatin) were isolated.

Organomercury Column Affinity Chromatography—Chicken erythrocyte chromatin fraction S150 was dialyzed against buffer A (10 mM Tris-HCl, pH 7.5, 25 mM KCl, 25 mM NaCl, 5 mM sodium butyrate, 0.1 mM phenylmethylsulfonyl fluoride, and 5 mM EDTA, pH 7.5) and then applied to an organomercurial column (Affi-Gel 501; Bio-Rad) that was pre-equilibrated with buffer A. The column (1.5 × 8 cm) was then washed with buffer A (flow rate, 60 ml/h) to remove unbound chromatin fragments until the absorbance at 260 nm returned to base line. This was followed by washing the column with 0.5 M NaCl in buffer A (buffer B) until the absorbance at 260 nm returned to base line. The bound nucleosomes were eluted by 10 mM DTT in buffer A. The released material was monitored by measuring absorbance at 260 nm (17). The fractions containing the unbound material and 0.5 M NaCl eluted material were pooled; the fractions eluted with DTT were pooled. For analysis of histones in the unbound and bound fractions, the fractions were acid-extracted by the addition of 4 N sulfuric acid to a final concentration of 0.4 N. Before lyophilization, the supernatants were dialyzed overnight against 0.1 M acetic acid and then against two changes of double-distilled H_2O.

Reaction of Nucleosomes with [^{3}H]Iodoacetic Acid—Ten μl/ml [^{3}H]iodoacetic acid (NEN Life Science Products, 204.9 mCi/mmol) containing 2.50 μCi was added to the chromatin fraction SE, S150, P150, and PE (2 A_{260}/ml) in buffer E (10 mM Tris-HCl, pH 8.2, 1 mM EDTA) and allowed to incubate at room temperature for 1 h in the dark. The chromatin fraction was applied directly to a hydroxylapatite column. The histones were isolated by acid extraction as described above. Histones were electrophoretically resolved on SDS-polyacrylamide gel electrophoresis. Following staining with Coomassie Blue, the gel pieces containing a histone band were disrupted in hydrogen peroxide and then counted in 5 ml of scintillation fluid.

Hydroxylapatite Chromatography—The chromatin fraction was mixed with hydroxylapatite HTP gel powder (Bio-Rad) at a ratio of 1 mg of DNA to 0.25 g of hydroxylapatite as described previously (27). The column was washed with 0.63 M NaCl in 0.1 M potassium phosphate buffer, pH 6.7, to remove histone H1, H5, and non-histone chromosomal proteins before applying a linear gradient of NaCl (0.63 to 2 M NaCl in 0.1 M potassium phosphate buffer, pH 6.7) at a flow rate of 35 ml/h as described previously (27).

DNA Preparation and Southern Blot Hybridization—DNA was prepared from the different chromatin fractions as described previously (24). For electrophoresis, equal amounts of DNA were dissolved in DNA sample loading buffer, and the samples were loaded onto 1% agarose minigels containing 0.5 μg of ethidium bromide/ml. The DNA was transferred to Hybond-N+ nylon transfer membrane and hybridized to radiolabeled probes as described previously (28). The cloned probes used were pCBG 14.4, a unique intronic sequence of chicken β globin gene; pChV2.5B/H, which contains the gene coding for chicken histone H5 and flanking sequences; and pVTG412, that recognizes the 5′ region of the chicken vitellogenin gene (24).

Polyacrylamide Gel Electrophoresis and Western Blotting—AUT (acetic acid, 6.7 M urea, 0.375% (w/v) Triton X-100) and SDS-15% polyacrylamide gel electrophoresis were performed as described elsewhere (24). Antiacetylated H3 and antiacetylated H4 antibodies generously supplied by Dr. D. Allis were used to detect acetylated species of H3 and H4 (29–31) in Western blot experiments using a protocol described previously (32).

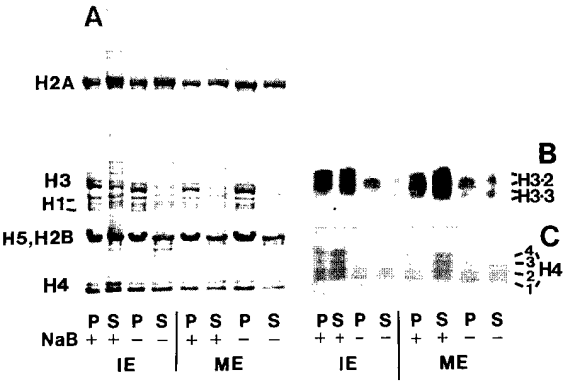

FIG. 1. **Level of acetylated H3 and H4 histones in immature and mature erythrocyte chromatin fractions S150 and PE.** *A*, histones were acid-extracted from chromatin fractions S150 (*S*) or PE (*P*) of immature (*IE*) or mature (*ME*) cells that were either untreated (−) or incubated in the presence of sodium butyrate for 1 h (+). The histones (9 μg) were electrophoretically resolved on an AUT-15% polyacrylamide gel. The gel was stained with Coomassie Blue. *B* and *C*, the proteins were electrophoretically transferred to nitrocellulose and immunochemically stained with antiacetylated H3 (*B*) or antiacetylated H4 (*C*) antibodies. The mono-, di-, tri-, and tetra-acetylated isoforms of H4 are marked as *1*, *2*, *3*, and *4*, respectively. Note that the content of H1 and H5 in fraction S150 is typically lower than that of the other chromatin fractions (65).

RESULTS

State of Acetylation of Dynamically Acetylated Histones in Chicken Immature and Mature Erythrocytes—Histone acetylation is rapidly reversible in immature erythrocytes (3, 4, 7). To find the steady state of acetylated H3 and H4, histones of immature and mature erythrocyte chromatin fractions S150 and PE were electrophoretically resolved on AUT-polyacrylamide gels, transferred to nitrocellulose, and immunochemically stained with either antiacetylated H4 antibodies or antiacetylated H3 antibodies. Both antibodies detect the multiacetylated forms of H4 and H3, with the antibody to acetylated H3 showing a strong preference for the highest acetylated isoforms of H3 (30). We have shown previously that highly acetylated histones are found in chromatin fractions S150 and PE, but not in fraction P150 (7, 24). Thus, this latter fraction was not analyzed. Fig. 1 shows that the steady state levels of highly acetylated H3 and H4 isoforms were low in fractions S150 and PE from immature erythrocytes. However, the steady state levels of the highly acetylated H3 and H4 isoforms in fractions S150 and PE were markedly increased when immature erythrocytes were incubated in the presence of sodium butyrate, a histone deacetylase inhibitor, for 60 min (Fig. 1). These results suggest that the rate of deacetylation is so rapid in immature erythrocytes that, once dynamically acetylated H3 and H4 reach a highly acetylated state, they are rapidly deacetylated

[2] The abbreviations used are: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; DTT, dithiothreitol.

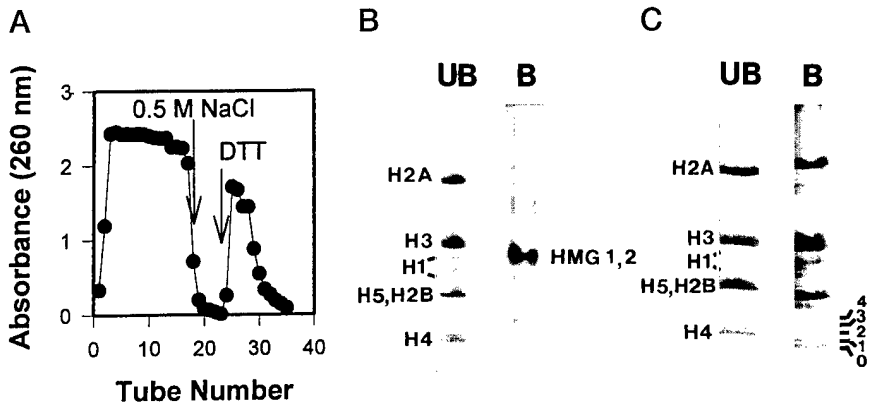

FIG. 2. **Fractionation of S150 fraction of immature erythrocyte chromatin by mercury affinity chromatography.** S150 chromatin fragments from immature erythrocytes or immature erythrocytes incubated with sodium butyrate were applied to a mercury-agarose column. Following the elution of unbound chromatin fragments, the column was washed with 0.5 M NaCl containing buffer. The mercury-bound chromatin fragments and proteins were released from the column by the addition of 10 mM DTT. The absorbance at 260 nm was monitored. *Panel A* shows the chromatograph of S150 (125 A_{260} units applied; 3-ml fractions collected) of butyrate-incubated cells. *Panels B* and *C*, the proteins (*UB*, 10 μg) of the unbound fractions including the 0.5 M NaCl wash fractions and the proteins (*B*, 10 μg) in the DTT-released fractions were electrophoretically resolved on AUT-15% polyacrylamide gels. The gels were stained with Coomassie Blue.

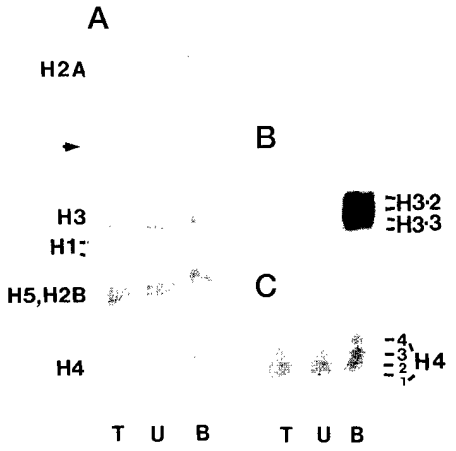

FIG. 3. **Analysis of acetylated H3 and H4 isoforms of immature erythrocyte chromatin fractionated by mercury affinity chromatography.** *A*, chromatin fraction S150 isolated from immature erythrocytes incubated with sodium butyrate was fractionated by mercury affinity chromatography. The proteins (15 μg) of fraction S150 (*T*), unbound (*UB*), and bound (*B*) fractions were electrophoretically resolved on an AUT-15% polyacrylamide gel. The gel was stained with Coomassie Blue. The protein indicated by the *arrow* comigrates with H1 on SDS-polyacrylamide gels. *B* and *C*, the proteins were electrophoretically transferred to nitrocellulose and immunochemically stained with antiacetylated H3 (*B*) or antiacetylated H4 (*C*) antibodies. The mono-, di-, tri-, and tetra-acetylated isoforms of H4 are marked as *1*, *2*, *3*, and *4*, respectively.

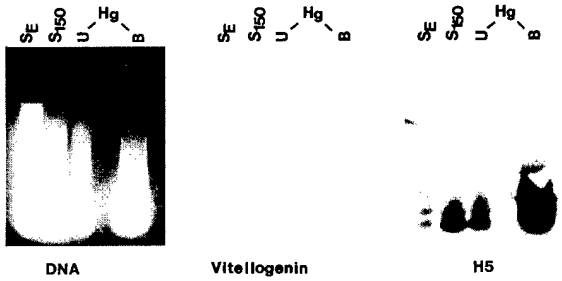

FIG. 4. **Analysis of DNA sequences of immature erythrocyte chromatin fractionated by mercury affinity chromatography.** Chromatin fraction S150 isolated from immature erythrocytes incubated with sodium butyrate was fractionated by mercury affinity chromatography. The DNA (10 μg) of chromatin fractions SE, S150, and mercury column unbound (*U*) and bound (*B*) fractions were electrophoretically resolved on a 1% agarose gel. The gel (*DNA*) was stained with ethidium bromide. The DNA was transferred to membranes and probed with either chicken vitellogenin (repressed) or histone H5 (active) DNA sequences.

(4); the net result is a low steady state level of highly acetylated histone isoforms in untreated immature cells.

The steady state of acetylated H3 and H4 in mature erythrocyte chromatin fractions was higher than that of H3 and H4 in the corresponding fractions of immature erythrocytes (Fig. 1). Incubation of mature cells with sodium butyrate elevated the level of hyperacetylated H3 and H4 isoforms in chromatin fraction S150. The level of highly acetylated H3 and, to a lesser extent, highly acetylated H4 in fraction PE was also increased.

Mercury-Agarose Column Fractionation of Salt-soluble Immature Erythrocyte Chromatin—The chromatin fraction S150 was isolated from chicken immature erythrocytes that were untreated or incubated with sodium butyrate for 90 min. The S150 chromatin fractions were applied to mercury-agarose columns (Fig. 2). Analysis of the proteins released from the mercury column with DTT showed that histones were present in the S150 chromatin fraction from butyrate incubated cells (Fig. 2*C*), but absent in the S150 fraction from untreated cells (Fig. 2*B*). The major proteins in the latter fraction were the cysteine-containing high mobility group proteins 1 and 2 (Fig. 2*B*). These results suggested that unfolded nucleosomes were absent or at very low levels in the S150 chromatin fraction from untreated immature erythrocytes. However, the unfolded nucleosome appeared to be present in immature erythroid cells that were incubated with butyrate. This result suggested that when deacetylation of dynamically acetylated histones was halted, the transcriptionally active nucleosomes was prevented from reverting to a thiol-nonreactive state.

To find if the mercury-agarose-bound nucleosomes from fraction S150 of butyrate-incubated immature erythrocytes had hyperacetylated histones, histones from S150, mercury-agarose-bound, and mercury-agarose-unbound chromatin fractions were analyzed in Western blot experiments with antiacetylated H3 and H4 antibodies. Fig. 3 shows that bound nucleosomes were enriched in hyperacetylated H3 and H4 isoforms.

DNA isolated from the S150, unbound and bound mercury-agarose chromatin fractions was analyzed by Southern blotting with probes containing DNA sequences to genes that were

either expressed or repressed in immature erythrocytes. The chromatin fraction bound to mercury-agarose contained transcriptionally active histone H5 and β-globin (not shown) DNA, but not repressed vitellogenin DNA (Fig. 4). These results show that mercury-bound nucleosomes were associated with transcriptionally active DNA.

Hydroxylapatite Dissociation Chromatography Analysis of Immature Erythrocyte-soluble and Nuclear Skeleton-associated Chromatin Labeled with [^{3}H]Iodoacetic Acid—The characterization of the thiol-reactive, unfolded nucleosome of transcribing chromatin described by Allfrey and colleagues has been done solely with soluble chromatin fragments. However, most transcribing chromatin is associated with the residual nuclear material (fraction PE), the nuclear skeleton. In immature erythrocytes approximately 75% of the transcribed DNA sequences are associated with the nuclear skeleton (7). To test the reactivity of the thiol group (Cys-110) of H3 in chromatin from butyrate-incubated immature erythrocytes, fraction SE and PE chromatin fragments were incubated with [^{3}H]iodoacetic acid. Fig. 5, *B* and *C*, shows that H3 was labeled in SE and PE chromatin. In contrast to the results obtained with fraction SE and PE chromatin, the H3 of chromatin fraction P150, which contained repressed DNA, was not labeled (data not shown).

To monitor the labeling of H3 in the chromatin fractions, hydroxylapatite dissociation chromatography was applied (27). Hydroxylapatite was added to fraction SE or PE in 0.63 M NaCl, removing non-histone chromosomal proteins and H1 histones from the hydroxylapatite-bound chromatin (27). Increasing concentrations of NaCl were then applied to the hydroxylapatite column, resulting first in the dissociation of H2A-H2B dimers followed by H3-H4 tetramers from the hydroxylapatite-bound chromatin (Fig. 6, *A* and *C*) (27). The interpeak fractions contained H2A, H2B, H3, and H4 (Fig. 6*C*, *lane b*). The concentration of NaCl required to dissociate the H2A-H2B dimer or H3-H4 tetramer from the nucleosomal DNA provides a measure of the strength of the interaction between the dimer or tetramer and DNA (27). For example, highly acetylated H3-H4 tetramers dissociate from hydroxylapatite-bound nucleosomal DNA at a lower ionic strength than do unmodified H3-H4 tetramers (27, 33).

Chromatin fragments of fractions SE and PE isolated from butyrate-incubated immature erythrocytes were incubated with [^{3}H]iodoacetate and then subjected to hydroxylapatite dissociation chromatography. Fig. 6, *A* and *B*, shows that labeled H3 dissociated from the hydroxylapatite-bound chromatin after the H2A-H2B dimers but before the bulk of the H3-H4 tetramers.

To determine whether incubation of immature erythrocytes with histone deacetylase inhibitors was required to detect the thiol-reactive H3 in nucleosomes, chromatin fractions SE and S150 were isolated from cells that were untreated or incubated with sodium butyrate. Following incubation with [^{3}H]iodoacetate, the chromatin fractions were applied to hydroxylapatite. Labeled H3 was detected only in fraction SE and S150 when cells were incubated with sodium butyrate (compare Fig. 6*A* with 7*C* and Fig. 7, *A* with *B*). Immature erythrocytes were also incubated with trichostatin A, a specific histone deacetylase inhibitor, instead of sodium butyrate. The dissociation profiles of hydroxylapatite bound SE chromatin fragments from trichostatin A-treated immature erythrocytes were similar to those from butyrate-treated cells (compare Fig. 7*D* with 6*A*).

Hydroxylapatite dissociation chromatography with PE chromatin was problematic as the addition of insoluble nuclear skeletons to hydroxylapatite reduced the flow rate appreciably. For the following experiments with the PE fraction, the suspension of nuclear skeletons was incubated with [^{3}H]iodoacetate, and then the acid-extracted histones were separated by SDS-gel electrophoresis. The H3 band was excised and counted (see Fig. 5). In accordance with the results with fraction SE and S150, H3 of fraction PE was thiol-reactive in chromatin fragments from butyrate-incubated, but not untreated, immature erythrocytes (Fig. 8). These observations suggest that the thiol reactivity of H3 in immature erythrocyte chromatin fractions

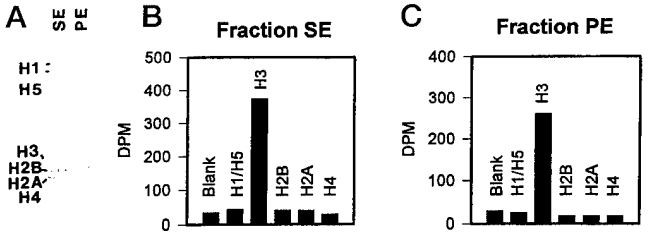

FIG. 5. **Detection of thiol-reactive H3 in SE and PE chromatin.** Chromatin fractions SE and PE were isolated from butyrate-incubated immature erythrocytes. The chromatin fraction was incubated with [^{3}H]iodoacetic acid. Histones (10 μg) acid extracted from SE and PE chromatin were electrophoretically resolved on a SDS-15% polyacrylamide gel. *Panel A* shows the Coomassie Blue-stained gel. The stained bands were excised and counted (*panel B*, fraction SE histones; *panel C*, fraction PE histones).

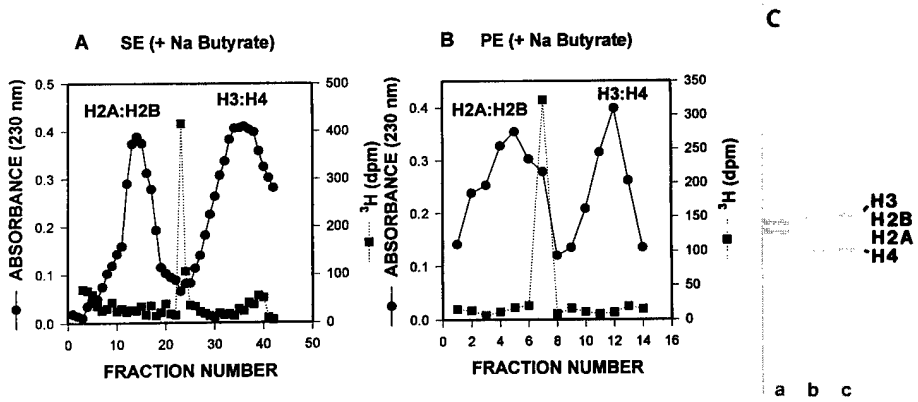

FIG. 6. **Hydroxylapatite dissociation chromatography of fraction SE and PE chromatin fragments of chicken immature erythrocytes.** Chromatin fraction SE (160 A_{260} units) and PE (226 A_{260} units) isolated from immature erythrocytes incubated with sodium butyrate (+ *Na Butyrate*) were incubated with [^{3}H]iodoacetate and then added to hydroxylapatite as described under "Experimental Procedures." For the chromatograms shown in *panels A* and *B*, fractions of 2 and 5 ml were collected, respectively. *Panel C*, chromatin fraction SE was applied to hydroxylapatite column, and fractions equivalent to fractions 8–17, 18–28, and 29–42 shown in *panel A* were pooled, dialyzed against water, and then lyophilized. The proteins (4 μg) from the three fractions (*lanes a*, *b*, and *c*, respectively) were electrophoretically resolved in a SDS-15% polyacrylamide gel. The gel was stained with Coomassie Blue.

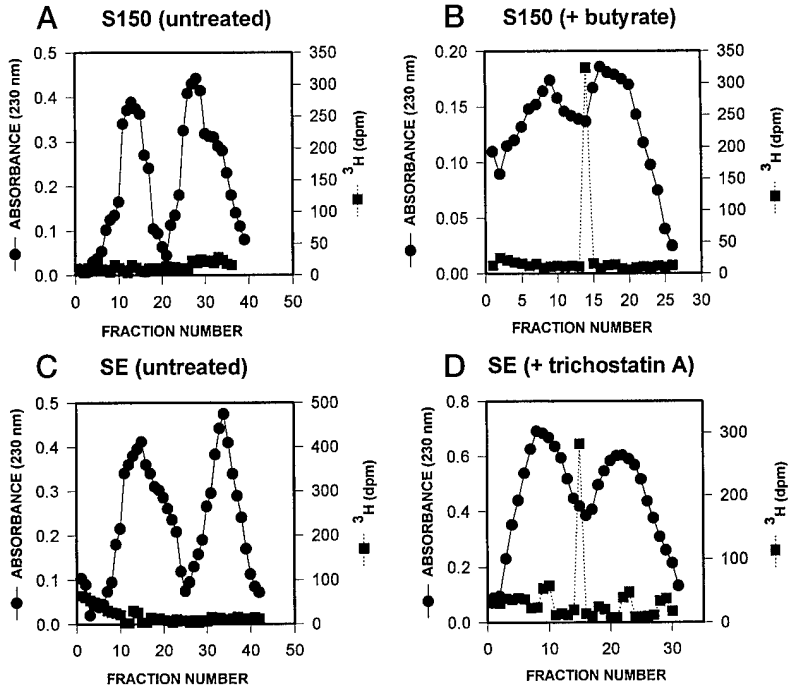

FIG. 7. **Hydroxylapatite dissociation chromatography of salt-soluble chromatin fragments of chicken immature erythrocytes.** *Panels A* and *B*, chromatin fraction S150 isolated from immature erythrocytes untreated (*panel A*, 160 A_{260} units) or incubated with sodium butyrate (*panel B*, 40 A_{260} units) was incubated with [^{3}H]iodoacetate and then added to hydroxylapatite as described under "Experimental Procedures." *Panels A* and *B*, 2- and 4-ml fractions were collected, respectively. *Panels C* and *D*, chromatin fraction SE isolated from immature erythrocytes untreated (*panel C*, 160 A_{260} units) or incubated with trichostatin A (*panel D*, 168 A_{260} units) was incubated with [^{3}H]iodoacetate and then applied to hydroxylapatite as described under "Experimental Procedures." Two-ml fractions were collected.

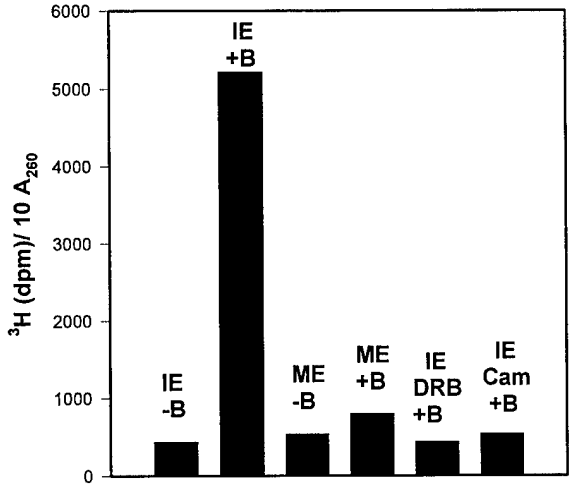

FIG. 8. **Thiol reactivity of H3 of chromatin fragments associated with the residual insoluble erythroid nuclear skeleton.** Chromatin fraction PE isolated from or immature erythrocytes (*IE*) or mature erythrocytes (*ME*) untreated (*−B*) or incubated with transcriptional inhibitors (DRB; camptothecin, *Cam*) and sodium butyrate (*+B*) was incubated with [^{3}H]iodoacetate. Acid-extracted histones were electrophoretically resolved on SDS-15% polyacrylamide gels. Histone H3 was excised and counted.

SE, S150, and PE is dependent upon the acetylation states of the nucleosomal histones.

Hydroxylapatite Dissociation Chromatography Analysis of Mature Erythrocyte Chromatin Labeled with [^{3}H]Iodoacetic Acid—Transcriptional elongation is arrested in mature erythrocytes. To find if hyperacetylating histones associated with transcriptionally competent chromatin (3, 34) was sufficient to observe a thiol-reactive H3, chromatin fractions S150 and PE from mature cells untreated or incubated with sodium butyrate were labeled with [^{3}H]iodoacetate. Figs. 8 and 9 show that labeled H3 was not observed in the mature erythrocyte S150 and PE chromatin fractions. These results and those with immature erythrocyte chromatin suggest that histone acetylation is required but not sufficient for formation and/or stabilization of nucleosomes with thiol-reactive H3.

Effect of Inhibitors of Transcription on the Thiol Reactivity of H3 in Nucleosomes—The absence of thiol-reactive nucleosomes in butyrate-treated mature erythrocytes indicates that both transcription elongation and highly acetylated histones are required to form and maintain the unfolded nucleosome conformation. To test whether inhibition of transcription elongation has an effect on the H3 thiol reactivity of immature erythrocyte nucleosomes, immature erythrocytes were incubated with inhibitors of transcription before the addition of sodium butyrate. Camptothecin is an inhibitor of topoisomerase I and has been reported to stimulate initiation but inhibit elongation by RNA polymerase II (26, 35, 36). A thiol-reactive H3 was not detected in the SE and PE chromatin of immature cells treated with DRB or camptothecin followed by butyrate (Figs. 8 and 10) (25). These results show that both highly acetylated histones and elongation are needed to detect the thiol-reactive H3 in immature erythrocyte chromatin.

DISCUSSION

We show that highly acetylated histones are required to maintain the unfolded, thiol-reactive structure of transcribing nucleosomes. The thiol-reactive nucleosome is not detected in the chromatin of transcriptionally active immature erythrocytes where the steady state level of highly acetylated histones is low. But when deacetylation of the highly acetylated histones is prevented by incubating immature erythrocytes with histone deacetylase inhibitors, the thiol-reactive nucleosome is detected. The rate of deacetylation in immature erythrocytes is such that the highly acetylated H3 and H4 isoforms are short lived (4). In contrast, the thiol-reactive nucleosome is detected in the chromatin of mammalian cells without the use of histone deacetylase inhibitors (37) (unpublished observations). These observations suggest that the net activities of the histone acetyltransferases and deacetylases decide the longevity of the unfolded nucleosome. In transcribing regions where the rate of histone deacetylation exceeds the rate of acetylation, the unfolded nucleosome structure will be short lived and will rapidly revert to a thiol nonreactive state following passage of the RNA polymerase. In yeast the converse is the case. The unfolded nucleosome structure associated with a specific gene persists well after the transcription of that gene has been arrested (38).

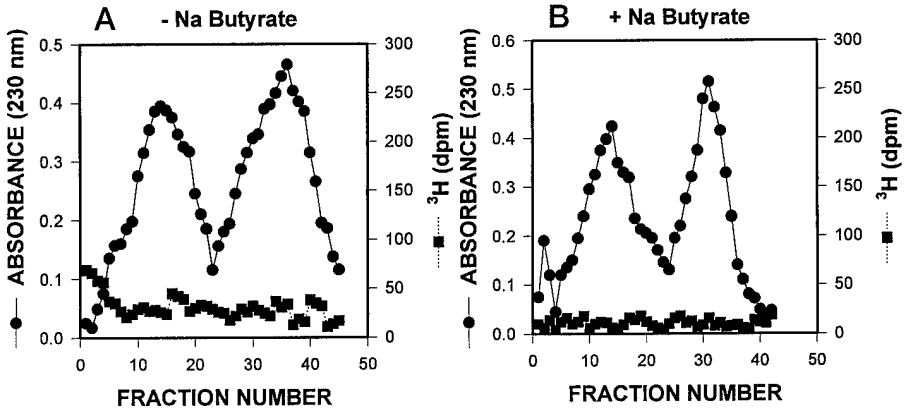

FIG. 9. **Hydroxylapatite dissociation chromatography of salt-soluble chromatin fragments of chicken mature erythrocytes.** Chromatin fraction S150 isolated from mature erythrocytes untreated (*panel A*, *− Na Butyrate*) or incubated with sodium butyrate (*panel B*, *+ Na Butyrate*) was incubated with [^{3}H]iodoacetate and then added to hydroxylapatite as described under "Experimental Procedures." *Panels A* and *B*, 160 A_{260} units of chromatin were added to hydroxylapatite; 2-ml fractions were collected.

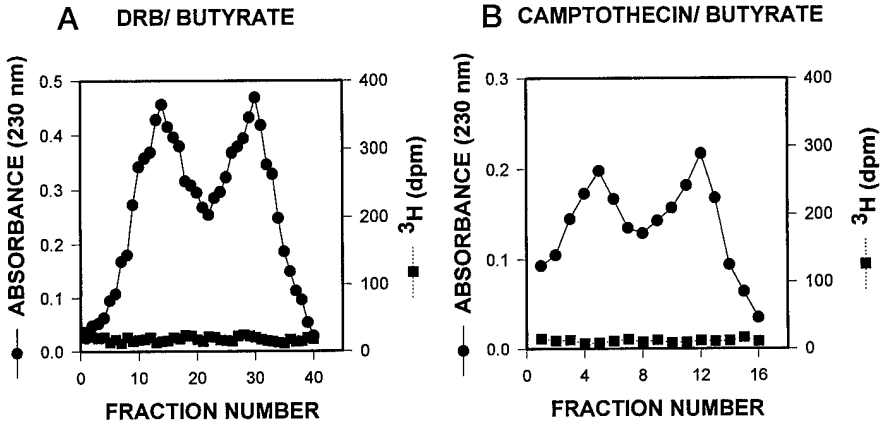

FIG. 10. **Hydroxylapatite dissociation chromatography of fraction SE chromatin fragments of chicken immature erythrocytes incubated with inhibitors of transcription elongation.** *Panel A*, and *B*, chromatin fractions SE were isolated from immature erythrocytes incubated with either DRB or camptothecin and then with sodium butyrate. The chromatin fractions were incubated with [^{3}H]iodoacetate and then added to hydroxylapatite as described under "Experimental Procedures." *Panel A*, 160 A_{260} units of chromatin applied; 2-ml fractions collected. *Panel B*, 60 A_{260} units of chromatin applied; 5-ml fractions collected.

The rates of histone acetylation and deacetylation are very slow in yeast, but the high steady state of highly acetylated histones argues that the rate of histone acetylation exceeds the rate of deacetylation (39, 40).

Histone acetylation, however, is not sufficient to generate the unfolded nucleosome structure; transcription elongation is required. Thiol-reactive nucleosomes could not be found in the chromatin of chicken mature erythrocytes. Transcription may be initiated in these mature cells, but RNA polymerases are paused at the 5′ end of the transcribed genes (41, 42). The treatment of immature erythrocytes with camptothecin may mimic the mature erythrocyte situation, that is, transcription initiation occurs but elongation does not (26). Thus, although dynamic histone acetylation and initiation are occurring in these cells at transcriptionally competent/active loci, without elongation the thiol-reactive nucleosome is not formed.

Our results suggest that unfolded transcribing nucleosomes are associated with highly acetylated H3-H4 tetramers. In agreement with the studies of Allfrey and colleagues (19), we found that immature erythrocyte chromatin fragments bound to mercury agarose are enriched in highly acetylated H3 and H4. Furthermore, Sterner *et al.* (20) showed that the thiol-reactive H3 of unfolded mammalian nucleosomes is hyperacetylated. Acetylation of H3 and H4 may maintain the unfolded nucleosome conformation by breaking interactions between the histone N-terminal tail and nucleosomal DNA. The N-terminal tail of H4 is not mobile in nucleosomes, and there is evidence that the H4 N-terminal tail makes intranucleosomal contacts (43). Indeed, His-18 in the N-terminal region of H4 cross-links to nucleotides 57, 66, and 93 from the 5′ end of nucleosomal DNA (44, 45). This position in the nucleosomal DNA corresponds to where the nucleosomal DNA is sharply bent or kinked. In active gene chromatin and in chromosomal domains containing hyperacetylated histones, the cross-linking between His-18 of H4 and nucleosomal DNA *in situ* is greatly diminished (44, 46, 47). Moreover, site 60 from the end of nucleosomal DNA of hyperacetylated nucleosomes has an increased susceptibility to DNase I (48). These observations strongly suggest that acetylation at lysines located in N-terminal tail of H4 may have important functions in altering histone-DNA contacts and nucleosome structure. Further, hyperacetylation of the H3-H4 tetramer reduces the linking number change per nucleosome, that is, negative DNA supercoils constrained in unmodified nucleosomes are partially released in nucleosomes with hyperacetylated histones (49, 50).

The destabilizing effect that histone acetylation has on H3-H4 tetramer-DNA interactions in transcribing nucleosomes is seen in hydroxylapatite dissociation chromatography. The H3 of thiol-reactive nucleosomes dissociated from hydroxylapatite bound S150 or SE chromatin after the dissociation of the H2A-H2B dimers but before the bulk of the H3-H4 tetramers. In a previous study we monitored the dissociation of labeled ([^{3}H]acetate) dynamically acetylated histones from hydroxylapatite-bound chromatin of immature erythrocytes. The dissociation of the labeled ([^{3}H]acetate) highly acetylated H3-H4 tetramers coincided exactly with that of labeled ([^{3}H]iodoacetate) H3 (27). These observations with SE chromatin from chicken immature erythrocytes suggest that the interaction between highly acetylated H3-H4 tetramer and DNA of transcribing nucleosomes is weaker than that of typical nucleosomes. Analysis of mercury-agarose bound nucleosomes by electron spectroscopic imaging also indicated that the H3-H4 tetramer of unfolded nucleosomes is disrupted (18). The disruption of the tetramer in transcribing nucleosomes may facilitate subsequent rounds of elongation.

Transcribing chromatin is associated with the insoluble residual nuclear material (fraction PE) which contains the nuclear matrix. The PE chromatin from butyrate treated immature erythrocytes had 76% of the active DNA and 74% of the acetate-labeled tetraacetylated H4 (7). Further, the PE fraction

retained 75–85% of the nuclear histone acetyltransferase and histone deacetylase activity (7, 13). The thiol-reactive nucleosome was detected in PE chromatin of butyrate-treated immature erythrocytes, but not in the PE chromatin of untreated immature erythrocytes or mature erythrocytes. Further, inhibition of transcription with camptothecin or DRB prevented the detection of the unfolded nucleosome. Thus, the results obtained with PE chromatin were similar to those observed with S150 or SE chromatin; both hyperacetylated histones and elongation are required to detect the unfolded nucleosome in fraction PE.

There is increasing evidence that the transcription machinery is associated with the nuclear matrix and that for chromatin to be transcribed it is spooled through the anchored large RNA polymerase complex (51–54). We have proposed that histone acetyltransferase and deacetylase are localized in these transcription foci (13, 14). Recent studies show that coactivators (CBP/p300, ACTR, and GCN5) and proteins associated with TATA-binding protein ($TAF_{II}250$) have histone acetyltransferase activity (55–60). Histone deacetylases (HDAC-1 and HDAC-2) are associated with corepressors (mSin3A and N-CoR) and the nuclear matrix bound transcription factor YY1 (61–64). These studies suggest that the basal transcription machinery and transcription factors recruit histone acetyltransferases and histone deacetylases to sites of transcription at the nuclear matrix. Nucleosome structure will be perturbed when the chromatin fiber is passed through the fixed RNA polymerase (transcriptosome) (53). While in a highly acetylated state, the unfolded nucleosome structure will persist, helping subsequent rounds of transcription. Our results are consistent with the idea that the nucleosome is a dynamic structure conforming its structure to facilitate movement of chromatin through the RNA polymerase II elongation complex, with dynamic histone acetylation having a major role in modulating the unfolded structure of transcribing nucleosomes.

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Nuclear matrix: application to diagnosis of cancer and role in transcription and modulation of chromatin structure

James R. Davie, Shanti Samuel, Virginia Spencer, Lydia Bajno, Jian-Min Sun, Hou Yu Chen, and Laurel T. Holth.

Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E OW3

Correspondence: James R. Davie Tel: 204-789-3369, Fax: 204-789-3900, E-mail: davie@cc.umanitoba.ca

Summary

The nuclear matrix is involved in the processing of the genetic information and in the organization of chromatin. In recent years we have come to appreciate the organization of functional domains within the nucleus (e.g., transcript domains, RNA processing sites, sites of replication). The nuclear matrix is the foundation from which this organization is built, providing a scaffold upon which nuclear processes such as DNA replication and transcription occur. Chromatin is arranged into loop domains through the attachment of matrix associated regions (MARs) at the base of the loop to nuclear matrix proteins. Considering the role of the nuclear matrix in the organization and processing of the genetic information, it is not surprising to find that nuclear matrix proteins are informative in distinguishing cell types and disease states. For example, nuclear matrix proteins informative in the diagnosis of cancer, including breast cancer have been identified. Typically nuclear matrices are obtained following the salt extraction of nuclease-digested nuclei. However, recent studies show that cisplatin preferentially crosslinks MAR DNA to nuclear matrix proteins *in situ*, providing a complimentary method to identify informative nuclear matrix proteins.

Transcribed, but not repressed chromatin, is associated with the nuclear matrix. Regions of a chromatin loop engaged in transcription are associated with the nuclear matrix through multiple dynamic interactions with nuclear matrix proteins. Nuclear matrix bound transcription factors, the transcription machinery and histone modifying enzymes are thought to mediate these dynamic attachments between the nuclear matrix and transcriptionally active chromatin. Core histones of transcriptionally active chromatin are dynamically acetylated, with histone acetyltransferases (HATs) and deacetylases (HDACs) catalyzing this reaction. Both of these enzymes, which are now known to be transcriptional coactivators and modulators, are associated with the nuclear matrix. We have proposed that these enzymes participate in the dynamic attachment of transcriptionally active chromatin with the nuclear matrix. Our recent studies show that cisplatin crosslinks nuclear matrix-bound transcription factors and transcription modulators to nuclear DNA *in situ*. This suggests that cisplatin will be most useful in the discovery of nuclear matrix MAR binding proteins involved in the organization of DNA and nuclear matrix bound transcription factors/modulators participating in the nuclear matrix association of transcriptionally active chromatin. (Supported by Medical Research Council of Canada)

I. Nuclear matrix isolation and structure

The nuclear matrix has a role in the organization and function of nuclear DNA. The structure of the nuclear

matrix consisting of residual nucleoli, surrounding nuclear pore-lamina complex, and internal matrix is revealed when nuclease-digested nuclei are extracted with salt (e.g., 0.25 M ammonium sulfate). The protocol that we use to isolate nuclear matrices is shown in **Fig. 1**. Briefly, nuclei are digested with DNAase I followed by extraction with 0.25 M ammonium sulfate, yielding NM1-IF [nuclear matrices (NM) with attached intermediate filaments (IF)] (Sun et al., 1994; Chen et al., 1996). Further extraction of the NM1-IF nuclear matrices with 2 M NaCl yields NM2-IF. The internal matrix of NM1-IF preparations has a fibrogranular appearance (Chen et al., 1996). Extraction of the NM1-IF with high salt removes proteins that decorate core filaments of the internal matrix (Penman, 1995; Nickerson et al., 1995). The core filament fiber network is also seen when nuclear DNA is removed from nuclease-digested cells by electroelution in solutions of physiological ionic strength (Jackson and Cook, 1988). Core filaments, composition of which is currently unknown, have a diameter of 10-13 nm. These filaments appear to be the underlying structure onto which other nuclear components are bound.

The nuclear matrix is composed of protein and RNA. The nuclear pore-lamina consists of lamins and pore proteins. The internal matrix has a complex protein composition, with heterogeneous nuclear ribonuclear proteins (hnRNP) being major components (Mattern et al., 1996). Most nuclear RNA is associated with the nuclear matrix and contributes to the structural integrity of the nuclear matrix (Nickerson et al., 1995). The absence of nuclear RNA may weaken nuclear matrix internal structures. For example, nuclear matrices isolated from chicken mature erythrocytes lack nuclear RNA and internal structures, while nuclear matrices from immature erythrocytes of anemic adult birds have internal structures and nuclear RNA (Chen et al., 1996).

II. Nuclear matrix proteins and the diagnosis of cancer

The protein composition of the nuclear matrix is both tissue and cell type specific, and undergoes changes with differentiation and transformation (Fey and Penman, 1988; Stuurman et al., 1990; Dworetzky et al., 1990; Cupo, 1991). Pathologists have long appreciated that irregular nuclear appearance is the signature of a malignant cell (Miller et al., 1992; Nickerson et al., 1995). Changes in the composition of nuclear matrix proteins in malignant cells may contribute to alterations in nuclear structure. Nuclear matrix proteins are informative markers of disease states (Khanuja et al., 1993; Keesee et al., 1994). Informative nuclear matrix proteins have been identified for bladder, breast, colon, prostate, head, and neck cancers (Getzenberg et al., 1991a;1996; Khanuja et al., 1993; Keesee et al., 1994; Donat et al., 1996). For example, the nuclear matrix protein PC-1 is found in the nuclear matrix proteins from prostate cancer but not in the nuclear matrix from normal prostate or benign prostatic hyperplasia (Getzenberg et al., 1991a). Recently, we reported that the nuclear matrix protein composition was radically altered in highly metastatic oncogene transformed mouse fibroblasts (Samuel et al., 1997b). Interestingly, highly metastatic *ras*-transformed 10T1/2 cells and highly metastatic *fes*-transformed NIH 3T3 cells had a similar set of nuclear matrix proteins that were not seen in poorly metastatic or non-tumorigenic parental mouse fibroblast cell lines. Clearly, this study shows a correlation between the nuclear matrix protein profile and the metastatic potential of the cell. Of potential importance is the demonstration that nuclear matrix proteins can be detected in the serum and urine of cancer patients, thus suggesting that the detection of specific nuclear matrix proteins may be of value in breast cancer diagnosis (Miller et al., 1992; Replogle-Schwab et al., 1996; Carpinito et al., 1996).

We have identified informative breast cancer nuclear matrix proteins (Samuel et al., 1997a). Typically we prepare NM2-IF nuclear matrices from breast cancer cell lines or breast tumours. To remove IFs from these preparations we disrupt nuclear matrices and attached IFs with urea (**Fig. 1**). The IFs are then allowed to reassemble and are removed from the soluble nuclear matrix proteins (Fey and Penman, 1988). Over a broad protein concentration range, this process is independent of protein concentration, but it is dependent upon temperature (**Fig. 2**). Performing the reconstitution at room temperature is recommended. About 8-10% of the nuclear protein is recovered in the nuclear matrix protein fraction.

In the search for informative breast cancer nuclear matrix proteins, we used human breast cancer cell lines T47D, MCF-7 and ZR-75 (ER+/hormone dependent), MDA MB231, and BT-20 (ER-/hormone independent), and T5-PRF (ER+/hormone independent). A non-tumorigenic, spontaneously immortalized human breast epithelial cell line known as MCF-10A1 (ER-/hormone independent) obtained from reduction mammoplasty was chosen as the closest representative of normal breast epithelia. Typically we isolate proteins from at least three nuclear matrix preparations of each cell line, and these proteins are electrophoretically separated on two dimensional gels. Comparative analysis of the two dimensional gel patterns identified nuclear matrix proteins of estrogen receptor (ER) positive breast cancer cells that were not found in ER- breast cancer cells or normal breast epithelial cells (Samuel et al., 1997a). Our criteria for designating a nuclear matrix protein as being informative in breast cancer was that the protein had to be present in each of the relevant preparations (either ER+ and/or ER- breast cancer cell nuclear matrix proteins), but not in the preparations of

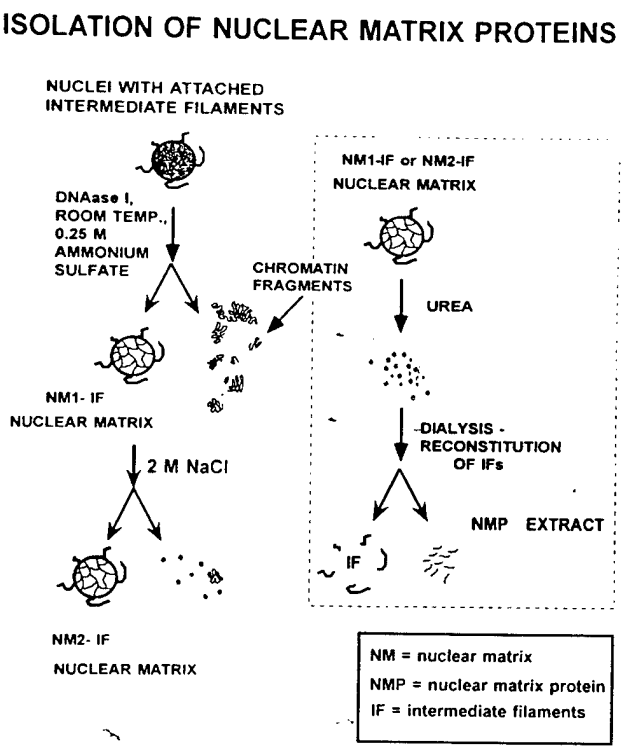

Figure 1. Method to Isolate Nuclear Matrix and Nuclear Matrix Proteins.

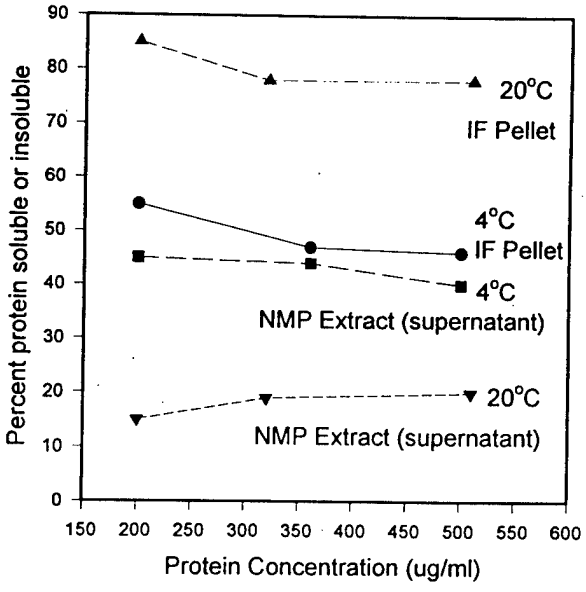

Figure 2. Effect of Temperature on the Separation of Intermediate Filament (IF) Proteins from Nuclear Matrix Proteins (NMP). NM2-IF from human breast cancer cells was disrupted in urea and then made to different protein concentrations prior to removal of IF proteins as described (Samuel et al., 1997b).

nuclear matrix proteins from "normal" breast epithelial cells. Using the nomenclature proposed by Khanuja et al. (1993), we refer to these proteins as NMBCs (nuclear matrix proteins in breast cancer). Five NMBCs (1-5) exclusive to the ER+ cell lines and one NMBC (6) exclusive to the ER- cell lines were identified (Samuel et al., 1997a).

The extracellular environment can alter the cellular morphology as well as the protein composition of the cytoskeletal and nuclear matrix compartments (Getzenberg et al., 1991b; Pienta et al., 1991; Fallaux et al., 1996). Thus, it was important to find out whether the changes in nuclear matrix proteins we observed with cancer cells grown on plastic were observed with cancer cells present in a breast tumour. In the preparation of nuclear matrices from breast tumours, we found that it was necessary to remove the adipose tissue surrounding the tumour. We found all NMBCs (1-5) exclusive to ER+ status in the human breast cancer cell lines as being present in the ER+ breast tumours, while NMBC-6 was not detectable (see **Fig. 3**, tumour 12797). NMBC-6, but not NMBCs 1-5, were present in ER- tumour nuclear matrix proteins.

The effect of cellular transformation on nuclear matrix protein composition is illustrated in the following study. Nuclear matrix proteins were isolated from MCF10A1 breast cancer cells that were transformed with the human T-24 mutated Ha-*ras* oncogene (MCF10AneoT) or with wild type human ER (cell line 139B6). MCF10AneoT cells are transformed and show anchorage independent growth (Basolo et al., 1991). The cell line 139B6 expresses ER at a similar level to that of MCF7 breast cancer cells (Pilat et al., 1996). In the presence of estradiol, this cell line has a slight inhibition in growth. This is typical of results of studies in which the ER is expressed in a ER- breast epithelial cell line or ER- breast cancer cell line (Pilat et al., 1996; Lundholt et al., 1996). Estradiol activated ER failed to elevate the expression of endogenous estrogen responsive genes but did induce the transient expression of an estrogen responsive element-regulated reporter gene in the 139B6 cell line (Pilat et al., 1996). Analysis of the two dimensional gel patterns of the nuclear matrix proteins from these cell lines revealed several alterations in nuclear matrix protein composition when MCF10A1 cells were transformed with *ras* or expressing ER. These differences were seen against a pattern of proteins found in all cell lines, for example hnRNP K (hk in **Fig. 3**). With the MCF10AneoT (Ha-*ras* transformed) cells, nuclear matrix proteins with a molecular mass of 47 kDa and pI range of 5.8-6.2 (constellation C in Fig. 3) were found to be exclusive to this cell line. Similarly, nuclear matrix proteins with molecular masses 50-57 kDa and pIs 5.5-5.7 (constellation B in **Fig. 3**) and proteins with molecular masses of 30-36

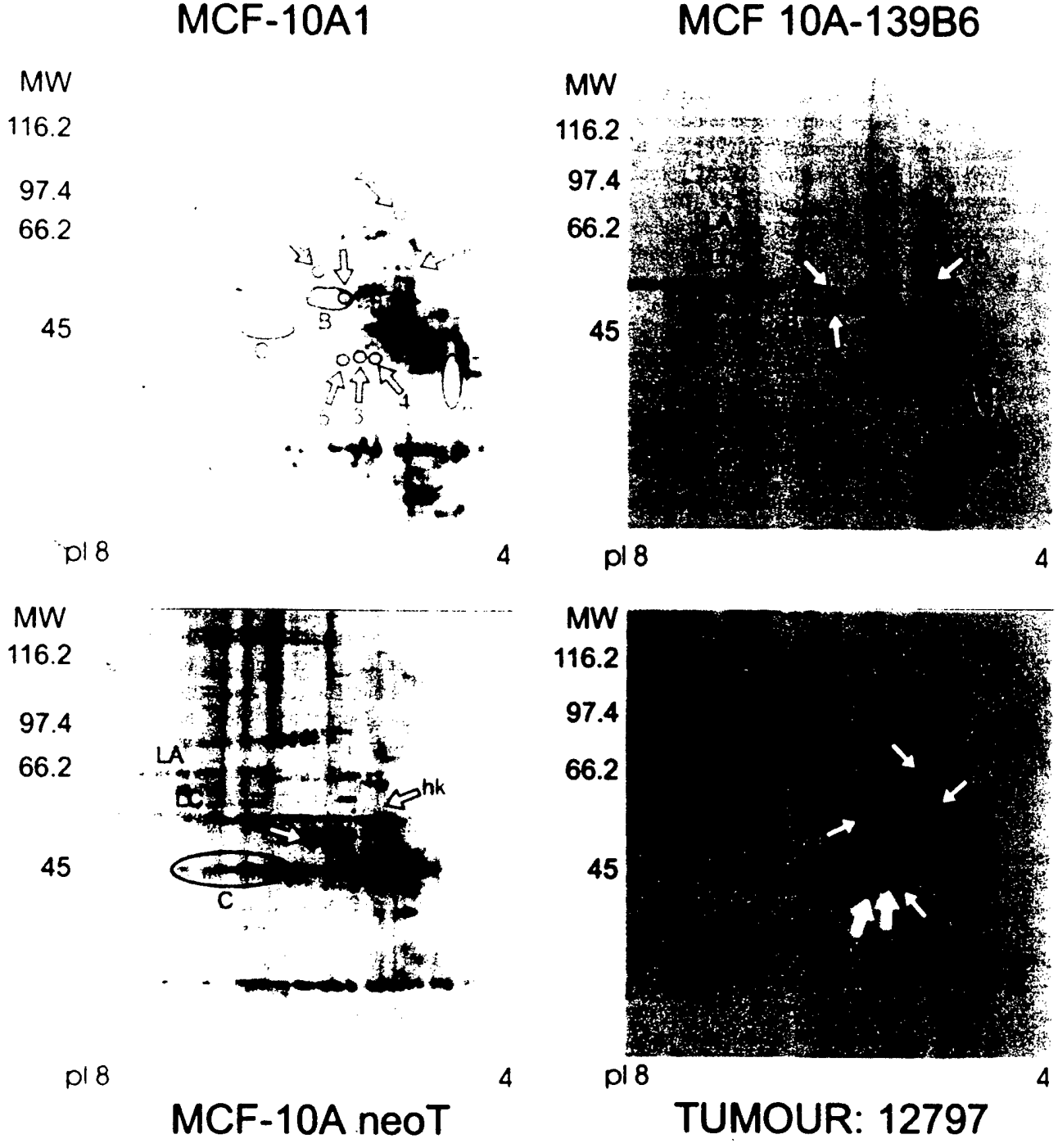

Figure 3. Human Breast Cancer Nuclear Matrix Proteins

Nuclear matrix proteins were isolated from MCF10A1 (parent, ER-, human breast epithelial cells), MCF10A-139B6 (parent transfected with human wild type ER), MCF10AneoT (parent transformed by T-24 Ha-*ras*), and human breast tumour 12797 (ER+). Protein (40 ug) was electrophoretically resolved on two-dimension gels. The gels were stained with silver. The position of the molecular weight standards (in thousands) is shown on the left side of each gel pattern. LA and LC are lamin A and C, respectively. The circles in MCF10A1 (parent) show the absence or decreased amount of nuclear matrix proteins highlighted in other gel patterns.

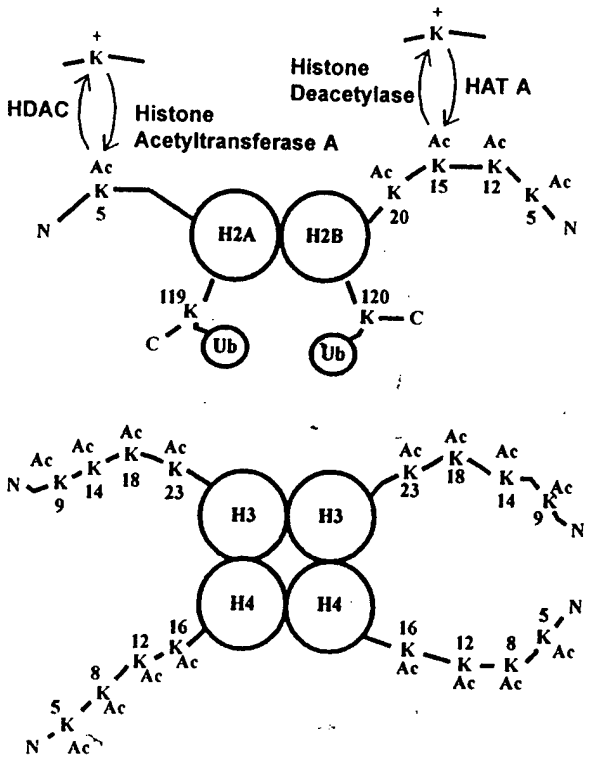

Figure 4. Sites of Post-Synthetic Modifications on the Histones.

The structures of H2A-H2B dimers and $(H3\text{-}H4)_2$ tetramers and the sites of modification are shown. Ac, acetylation; Ub, ubiquitination. The enzymes catalyzing reversible histone acetylation are shown.

kDa and pIs 4.5 (constellation A in **Fig. 3**) were determined to be exclusive to the ER expressing cell line MCF10A-139B6. However, within constellation B, a 48 kDa (pI 5.5) nuclear matrix protein (denoted by * in **Fig. 3**) was observed in the MCF10A parent cell line as well as in the *ras-* transformed and ER transfected cell lines. Relative to the parent cell line, the level of this protein in MCF10AneoT and MCF10A-139B6 was higher. NMBC1 present in the ER+ breast tumor nuclear matrix proteins was also detected in the ER expressing cell line (**Fig. 3**). The presence of NMBC1 in the ER transfected cell line suggests that ER expression has a role in the association of NMBC1 with the nuclear matrix. These results illustrate how nuclear matrix protein profiles reflect alterations in a cell's physiological state.

III. Nuclear matrix and organization of nuclear DNA

Nuclear DNA is packaged into nucleosomes, the repeating structural units in chromatin (Van Holde, 1988). The nucleosome consists of an histone octamer core around which DNA is wrapped. The four core histones of the octamer are arranged as a $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers positioned on both sides of the tetramer. The core histones have a similar structure with a basic N terminal unstructured domain, a globular domain organized by the histone fold, and a C terminal unstructured tail (Arents and Moudrianakis, 1995) (**Fig. 4**). Histone H1 binds to the linker DNA, which joins nucleosomes together, and to core histones (Boulikas et al., 1980; Banères et al., 1994). H1 has a tripartite structure with a basic N terminal domain, a basic C terminal tail domain, and a central globular core (Ramakrishnan, 1994).

In low ionic strength, chromatin fibers depleted of H1 have a "beads-on-a-string" structure, but with H1, folding of the fiber is evident (Leuba et al., 1994). At physiological ionic strength chromatin is folded into a 30 nm fiber. H1 stabilizes the folding of the chromatin fiber (Shen et al., 1995). The native 30 nm chromatin fiber has an irregular structure (an irregular three dimension zigzag) *in vitro* (Woodcock and Horowitz, 1995). Woodcock and colleagues show that the irregularities of the 30 nm chromatin fiber can be accurately reflected in a model that accounts for variability in linker DNA length and angle of trajectory that the linker DNA has as it enters and leaves the nucleosome. Thirty nm fibers are usually not seen inside nuclei (Woodcock and Horowitz, 1995). The chromatin is observed as matted patches. It appears that neighboring zigzags interdigitate, preventing individual chromatin fibers from being seen in nuclei. The core histone tails contribute to the condensation of the chromatin fiber (Garcia-Ramirez et al., 1995; Schwarz et al., 1996; Krajewski and Ausió, 1996). H3 and H4 tails are needed for fiber-fiber interactions (Schwarz et al., 1996).

The chromatin fiber is organized into loop domains, with an average size of 86 kb (Jackson et al., 1990; Gerdes et al., 1994) (**Fig. 5**). Transcriptionally active genes are found in DNAase I-sensitive, presumably decondensed chromatin loops that are accessible to transcription factors and transcription machinery (Davie, 1995). Transcriptionally inactive genes are in higher order, interdigitated chromatin patches, being essentially invisible to transcription factors and the transcription machinery. At the base of the loop there are DNA sequences called MARs (matrix associated regions) that bind to nuclear matrix proteins (Bode et al., 1995). MARs tend to be AT-rich, but do not have a consensus sequence (Bode et al., 1995; Mielke et al., 1996). MAR-DNA binds to both internal matrix and nuclear pore-lamina, suggesting that proteins of the nuclear pore-lamina and internal matrix are involved in the organization of chroma-

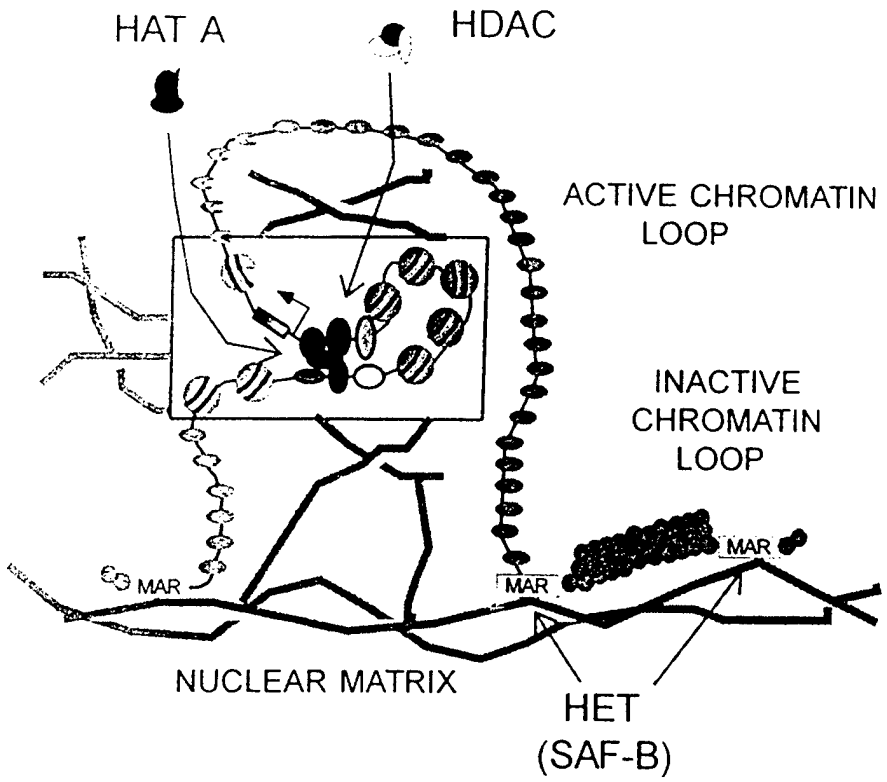

Figure 5. A Model for Transcriptionally Active and Repressed Chromatin Domains

At the base of the loop are nuclear matrix associated regions (MARs). HET (SAF-B) is a nuclear matrix protein that binds MARs. The repressed chromatin loop has a condensed chromatin structure. Multiple dynamic attachment sites between the transcriptionally active domain and the internal nuclear matrix are presented in the box outline. Histone acetyltransferase (HAT A), histone deacetylase (HDAC), transcription machinery and transcription factors are shown associated with the internal nuclear matrix, mediating a dynamic attachment between transcriptionally active chromatin and the nuclear matrix. HAT A and HDAC are shown as multiprotein complexes.

tin (Zini et al., 1989). MAR-binding proteins include lamins, which are found in the nuclear pore-lamina and internal matrix (Hozák et al., 1995), topoisomerase II, SATB1, HET (SAF-B), and attachment region binding protein which is an internal matrix protein or nuclear matrin (Pommier et al., 1990; Nakayasu and Berezney, 1991; von Kries et al., 1991; Luderus et al., 1992; Nakagomi et al., 1994; Buhrmester et al., 1995; Oesterreich et al., 1997).

Alterations in MAR-binding proteins have been reported in cancer cells. In Southwestern blotting experiments with a radiolabelled mouse IgH MAR sequence, Yanagisawa et al. (1996) detected a 114-kDa MAR binding protein expressed in breast carcinomas but not normal or benign breast tissue. Further, the levels of this MAR-binding protein were elevated in poorly differentiated breast ductal carcinomas. A recent study shows that mutant, but not wild type, p53 binds to MARs (Müller et al., 1996). Changes in nuclear matrix, MAR-binding proteins could result in reorganization of nuclear DNA.

IV. *In situ* crosslinking with cisplatin

Recent studies suggest that cisplatin (*cis*-diammine dichloroplatinum or *cis*-DDP) preferentially crosslink MARs to nuclear matrix proteins *in situ*. Either cells or nuclei can be incubated with cisplatin to crosslink protein to DNA. Most proteins crosslinked to DNA with cisplatin are nuclear matrix proteins, and the DNA crosslinked to protein is enriched in MAR-DNA sequences (Wedrychowski et al., 1986; 1989; Ferraro et al., 1992; 1995; Bubley et al., 1996; Olinski et al., 1987). **Fig. 6** shows the protocol to isolate proteins crosslinked to DNA *in situ*. A comparison of two dimension gel patterns of nuclear matrix proteins and proteins crosslinked to DNA with cisplatin in ZR-75 human breast cancer cells shows that several abundant nuclear matrix proteins are crosslinked to DNA in the cells (**Fig. 7**). Lamins A and C, components of the nuclear pore-lamina, are crosslinked *in situ* to nuclear DNA consistent with *in vitro* data suggesting that these proteins are involved in the organization of nuclear DNA (Wedrychowski et al., 1986; 1989). Abundant nuclear matrix proteins found

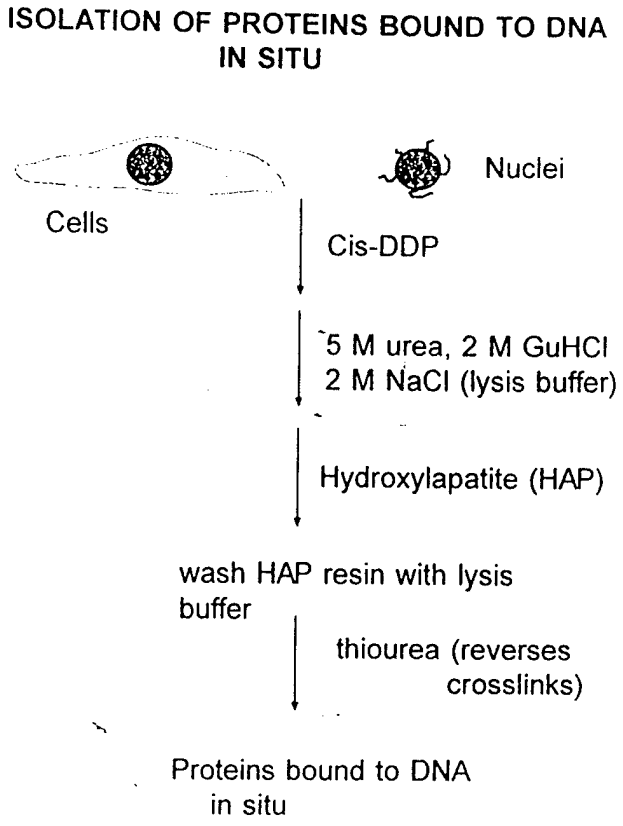

Figure 6. Method to Isolate Proteins Crosslinked to DNA in Cells or Nuclei with Cisplatin

crosslinked to nuclear DNA *in situ* with cisplatin are F-actin and hnRNP K (Miller et al., 1991; Sauman and Berry, 1994) (**Fig. 7**). HnRNP K is a single-strand DNA-binding protein that is associated with the nuclear matrix and has an important role in regulating the expression of the c-*myc* gene (Michelotti et al., 1996; Mattern et al., 1996). Further, hnRNP K interacts with TATA-binding protein (Michelotti et al., 1996). This transcription factor is a prominent protein observed in both the nuclear matrix fraction and proteins crosslinked to DNA *in situ* with cisplatin in ZR-75 human breast cancer cells (**Fig. 7**).

The ability of cisplatin to preferentially crosslink nuclear matrix proteins to nuclear DNA *in situ* has great potential in identifying nuclear matrix proteins involved in the organization and function of nuclear DNA. Several transcription factors are nuclear matrix proteins thought to interact with promoter and enhancer elements of specific genes. It has been proposed that the interaction of nuclear matrix bound transcription factors with regulatory DNA sequences has a role in attaching transcriptionally active chromatin to nuclear matrix (see below). Crosslinking with cisplatin may provide a method to find if the nuclear matrix associated transcription factor is bound to the DNA sequence of interest *in situ*. We are currently developing methods that will identify nuclear matrix associated transcription factors and their bound DNA sequences. Further, these methods are being used to find informative DNA-binding nuclear matrix proteins in the diagnosis of cancer.

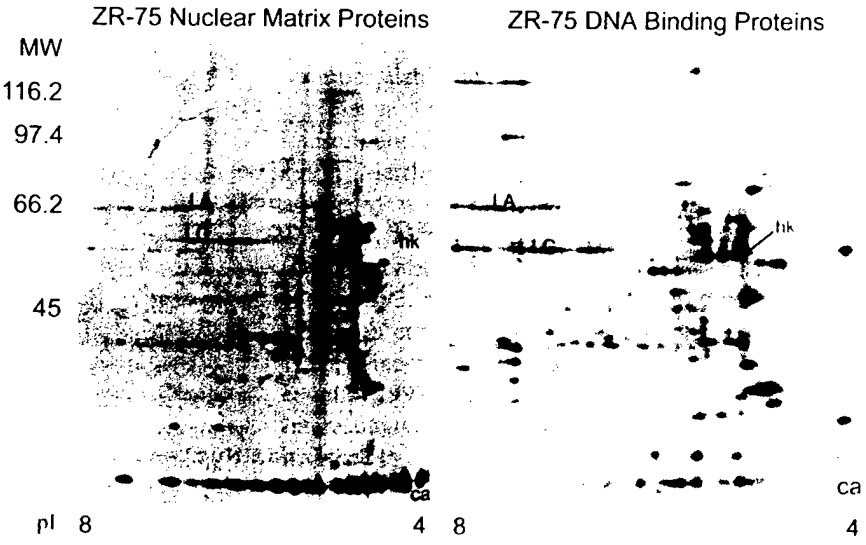

Figure 7. Analysis of Nuclear Matrix Proteins and Proteins Crosslinked to DNA

ZR-75 human breast cancer nuclear matrix proteins (40 ug) and proteins crosslinked to DNA by *cis*-DDP *in situ* (40 ug) were electrophoretically resolved on two dimension gels. The gels were stained with silver. The position of the carbamylated forms of carbonic anhydrase is indicated by ca. The position of the molecular weight standards (in thousands) is shown to the left of the gel patterns. LA and LC show lamin A and C, respectively. HnRNP K is shown as hk.

V. Transcription factors: activators of transcription

Current evidence suggest that an interaction between an enhancer or locus control region and promoter is an essential step in forming the open chromatin domain (Reitman et al., 1993). The enhancer/locus control region-promoter interaction is mediated by protein-protein associations between transcription factors bound to these *cis*-acting regulatory elements. This complex recruits the transcription initiation machinery and initiates the transcription cycle. The transcription cycle can be separated into at least four stages: initiation, promoter clearance, elongation, and termination. During the initiation stage, the pre-initiation complex (PIC) is formed at the promoter of a RNA polymerase II transcribed gene. The basal transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH and RNA polymerase II are involved in the assembly of the PIC (for review see Orphanides et al., 1996; Pugh, 1996; Nikolov and Burley, 1997). *In vitro* studies show that there is a defined order by which the basal transcription factors are assembled into the PIC. TFIID, one of the first factors involved, binds to the TATA-box and consists of the TATA-binding protein (TBP) and several TBP-associated factors (TAFs). However, there is evidence that the PIC comes partially preassembled (Maldonado et al., 1996). In the formation of the PIC at any given promoter, TFIID binding appears to be the rate limiting step. Transcription factors bound to enhancers and upstream promoter elements interact through their activation domains with the TAFs of TFIID or with other components of the PIC (e.g., TFIIB), increasing the rate that the PIC is formed at the promoter (for review see Tjian and Maniatis, 1994; Chiang et al., 1996; Gupta et al., 1996). TAFs are referred to as coactivators, proteins that mediate an interaction between transcription factors and PIC. Transcription factors bind to different TAFs, and these multiple contacts between the transcription factors and TAFs ensure the efficient recruitment of TFIID (Sauer et al., 1995; Chi et al., 1995). Transcription factors with multimerization domains also have key roles in juxtapositioning enhancer and promoter elements. For example, multimers of the transcription factor Sp1 bound at DNA sites separated by 1.8 kb will interact, resulting in a looping out of the intervening sequences (Pascal and Tjian, 1991). Once these *cis*-acting elements are positioned next to each other, there will be a high local concentration of activators in the vicinity of the promoter (**Fig. 8**).

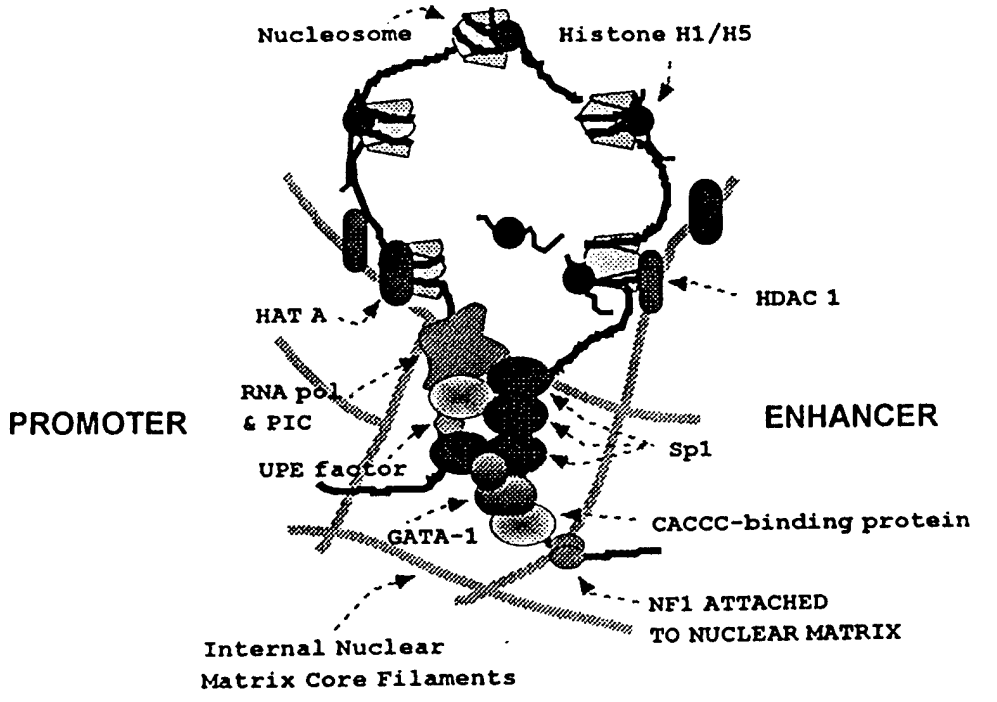

Figure 8. Model for Histone H5 Chromatin in Chicken Immature Erythrocytes.

The 3' enhancer is positioned next to the 5' promoter through protein-protein interactions. NF1 and multiprotein complexes (HAT As, HDACs, transcription machinery) are shown mediating the dynamic attachments of the histone H5 gene to the nuclear matrix at sites of transcription.

VI. Regulation of transcription

Within the DNAase I sensitive chromatin domains containing transcriptionally active genes are regions that are hypersensitive to DNAase I attack. The DNAase I hypersensitive (DH) regions of chromatin may lack nucleosomes and often mark chromatin for the presence of *cis*-acting regulatory DNA sequences and *trans*-acting factors. DH sites in human breast cancer c-*myc* chromatin and chicken erythrocyte histone H5 chromatin map with promoters and enhancers, and using *in vitro* assays we identified the transcription factors binding to these regulatory DNA elements (Penner and Davie, 1992; 1994; Sun et al., 1992; 1993; Miller et al., 1993; 1996; Murphy et al., 1996). However, *in vitro* assays can sometimes be misleading, and the most rigorous method in finding transcription factor occupancy is *in situ* footprinting (Becker et al., 1987; Mueller and Wold, 1989). We did *in situ* footprinting using a procedure called ligation-mediated PCR to reveal the occupancy of factor binding sites in the promoter and enhancer of the H5 gene in chicken erythrocytes (Sun et al., 1996b). Some factor binding sites in the promoter and enhancer identified *in vitro* were not occupied *in situ*. Based upon our studies on the chromatin structure and transcription factors associated with the H5 promoter and enhancer, we put forth a model for the transcriptionally active H5 gene (**Fig. 8**).

VII. Nuclear matrix and processing of the genetic information

The nuclear matrix is involved in the processing of the genetic information. In recent years we have come to appreciate that functional components (e.g., transcript domains, RNA processing sites, sites of replication) of the nucleus are highly organized (Hendzel and Bazett-Jones, 1995; Penman, 1995; Xing et al., 1995). Transcribed genes are found in discrete foci (Jackson et al., 1993; Iborra et al., 1996; Wansink et al., 1996). The nuclear matrix is the foundation from which this organization is built, providing a scaffold from which nuclear processes such as DNA replication and transcription occur (Berezney, 1991; Iborra et al., 1996). It is important to note that these functional centers in the nucleus are dynamic in their formation and dissociation. For example, sites of replication will assemble on the nuclear matrix at or near transcription foci in early S phase of the cell cycle. Once replication of these regions of the genome is complete, the replication machinery will disassemble from its site on the nuclear matrix and reassemble at other sites continuing replication of other regions of the genome (Hozák et al., 1993; Bassim Hassan et al., 1994). The process of transcription occurs at the nuclear matrix, and it has been proposed that the chromatin fiber moves through the nuclear matrix bound transcription apparatus as transcription proceeds (Cook, 1994; Hendzel and Bazett-Jones, 1995; Iborra et al., 1996). The transcription machinery is a massive multiprotein complex (Aso et al., 1995; Chao et al., 1996; Maldonado et al., 1996). Thus, it is unlikely that RNA polymerase travels along the DNA as text book models often show. Further, the nascent RNA becomes associated with the nuclear matrix. A solid state process by which DNA is driven through the nuclear matrix bound machinery and the nascent RNA is processed at the nuclear matrix would be an efficient way of dealing with these nuclear activities.

VIII. Nuclear matrix and transcriptionally active chromatin

Transcribed and nontranscribed sequences are precisely compartmentalized within the nucleus (Andreeva et al., 1992; Gerdes et al., 1994; Davie, 1995). Actively transcribed, but not inactive, chromatin regions are immobilized on the nuclear matrix by multiple dynamic attachment sites (**Fig. 5** and **8**). When histones are removed by high salt, loops of DNA are seen emanating from a central nuclear skeleton, forming a halo around this nuclear structure. Transcriptionally inactive genes are found in the halo, while DNA loops with transcriptionally active genes remain associated with the nuclear skeleton (Gerdes et al., 1994). The transcription machinery, specific transcription factors, and nuclear enzymes (e.g., histone acetyltransferase, histone deacetylase, see **Fig. 8**) are thought to mediate the dynamic attachments between transcribing chromatin and nuclear matrix (van Wijnen et al., 1993; Cook, 1994; 1995; Bagchi et al., 1995; Merriman et al., 1995).

The nuclear matrix is selective for which transcription factors it binds, and this selectivity varies with cell type (van Wijnen et al., 1993; Sun et al., 1994; 1996a). It has been postulated that the nuclear matrix has a role in the expression of genes by concentrating a subset of transcription factors at specific nuclear sites (Stein et al., 1991; Merriman et al., 1995). Transcription factors associated with the nuclear matrix include ER, HET, GATA-1, YY1, AML-1, Sp1, Oct1, mutant p53, and Rb (Dworetzky et al., 1992; Isomura et al., 1992; Vassetzky et al., 1993; van Wijnen et al., 1993; Sun et al., 1994; Merriman et al., 1995; Guo et al., 1995; Müller et al., 1996; Mancini et al., 1996; Kim et al., 1996; Oesterreich et al., 1997). HET is a transcriptional repressor. Interestingly, sequencing of HET revealed that it was identical to SAF-B, a protein isolated by its ability to bind MARs (Renz and Fackelmayer, 1996; Oesterreich et al., 1997). Thus, HET (alias SAF-B) is a nuclear matrix protein in breast cancer cells that binds to MARs and acts as a repressor.

Protein domains involved in targeting transcription factors to the nuclear matrix have been identified. However, it is too early to know whether a consensus nuclear matrix localization signal will emerge from these studies. The N-terminal domains of the androgen and glucocorticoid receptor are involved in directing these receptors to the nuclear matrix (van Steensel et al., 1995). There are examples of where the association of transcription factors with the nuclear matrix is regulated by modifications. For example, the association of Rb with the nuclear matrix appears to be regulated by phosphorylation and is cell-cycle dependent. When Rb is in a hypophosphorylated state in G1-phase of the cell cycle, it is attached to the nuclear matrix. But the highly phosphorylated Rb of S-phase is not associated with the matrix (Mancini et al., 1994). The amino terminus of hypophosphorylated Rb binds to a p84 nuclear matrix protein (Durfee et al., 1994).

ER is associated with the nuclear matrix of estrogen responsive tissues (Metzger and Korach, 1990; Metzger et al., 1991; Thorburn and Knowland, 1993). *In vitro* reconstitution studies with nuclear matrices and hormone receptors (e.g., ER and androgen receptor) show that nuclear acceptor sites for the hormone receptors are associated with the nuclear matrix (Barrack, 1987; Metzger and Korach, 1990; Lauber et al., 1995). The binding of the ER to the nuclear matrix was saturable, of high affinity, target tissue specific, and receptor specific (Metzger and Korach, 1990). Acceptor proteins for ER have been identified in a variety of estrogen-responsive tissues (Lauber et al., 1995; Ruh et al., 1996).

Transcription factors associated with the nuclear matrix can change throughout development and differentiation (Stein et al., 1994; Davie, 1995; Merriman et al., 1995; Bagchi et al., 1995; Sun et al., 1996a). For example, transcription factors associated with the chick erythrocyte nuclear matrix change throughout development (Sun et al., 1996a). Primitive red blood cells from 5-day old embryos have high levels of nuclear matrix-bound transcription factors, including GATA-1, CACCC-binding proteins, and NF1; factors that have key roles in erythroid-specific gene expression. In definitive red blood cells (11-day and 15-day embryos) the levels of these nuclear matrix bound transcription factors decline. Erythroid nuclear matrices preferred to bind CACCC-binding proteins and not Sp1. Promoters and enhancers of erythroid-specific genes have Sp1 binding sites that bind both CACCC-binding proteins and Sp1. It is possible that the selective nuclear matrix binding of CACCC-binding proteins gives the CACCC-binding proteins an advantage over Sp1 in binding to a Sp1/CACCC site.

Although we know that transcription factors are associated with the nuclear matrix, evidence that nuclear matrix associated transcription factors are bound to regulatory DNA elements of specific genes is currently lacking. For example, NF1 is a nuclear matrix associated transcription factor that binds to the enhancer of the chicken histone H5 gene (**Fig. 8**). *In vitro* footprinting and electrophoretic mobility shift assays show that NF1 isolated from immature erythrocyte nuclear matrices binds to the H5 enhancer (Sun et al., 1994). *In situ* footprinting shows that the NF1 binding site in the H5 enhancer is occupied in chicken immature erythrocytes (Sun et al., 1996b). We have proposed that NF1 recruits the H5 enhancer to the nuclear matrix (Davie, 1996). However, we have yet to show that nuclear matrix associated NF1 is the protein occupying the H5 enhancer NF1 binding site in erythroid cells. Cisplatin crosslinking may provide direct evidence to test this model (see above).

IX. Dynamic histone acetylation

Transcribed DNA is associated with acetylated histones (Hebbes et al., 1994; O'Neill and Turner, 1995; Mutskov et al., 1996). The core histones are reversibly modified by acetylation of lysines located in their basic N terminal domains (**Fig. 4**). Reversible histone acetylation is catalyzed by histone acetyltransferases (HATs) and deacetylases (HDACs), with the level of acetylation being decided by the net activities of these two enzymes. Histone acetylation alters nucleosome and higher order chromatin structure (Davie, 1995; 1997). For example, chromatin associated with highly acetylated histones does not undergo histone H1 mediated aggregation at physiological ionic strength, while chromatin with unacetylated histones aggregates when associated with H1 (Ridsdale et al., 1990; Davie, 1997). Besides modulating nucleosome and higher order chromatin packaging, the core histone tails bind to regulatory proteins (Ma et al., 1996; Edmondson et al., 1996). For example, yeast repressor protein Tup1 binds to the tails of H3 and H4. Acetylation of H3 and H4 prevents the binding of Tup1 (Edmondson et al., 1996). In mammalian cells and chicken erythrocytes, transcriptionally active chromatin regions have core histones undergoing high rates of acetylation and deacetylation, while in repressed chromatin regions the rate of reversible acetylation is slow (Davie, 1996; 1997). Thus, we expect that the interaction of regulatory proteins with the histone tails and chromatin structure of transcriptionally active regions of mammalian and chicken erythrocytes is in dynamic flux.

The process of reversible histone acetylation is not dependent upon ongoing transcription (Ruiz-Carrillo et al., 1976). To date, the only histone modifications dependent upon ongoing transcription are ubiquitination of H2B (see **Fig. 4**) and phosphorylation of mouse H1b (Davie and Murphy, 1990; Chadee et al., 1997). However, interference of dynamic acetylation by inhibiting

deacetylation with histone deacetylase inhibitors (e.g., sodium butyrate, trichostatin A or trapoxin) greatly affects cell cycle progression, arresting cells in G1 or G2, and may enhance or repress the expression of genes (Yoshida et al., 1995; Johnston et al., 1992; Girardot et al., 1994; Miyashita et al., 1994; Laughlin et al., 1995).

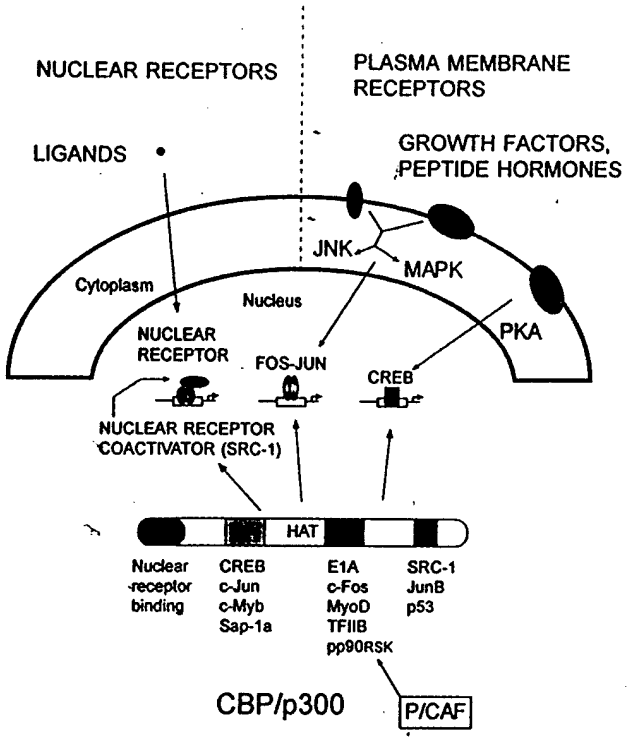

Figure 9. CBP/p300 Cointegrates Diverse Signalling Pathways

CBP and its functional/protein interaction domains are shown.

X. Histone acetyltransferase and gene activation

Histone acetylation is not limited to transcriptionally active chromatin, but also has a role in DNA replication (deposition-related acetylation) and DNA repair (for review see Davie, 1995; 1997). Deposition-related acetylation of H4 is catalyzed by HAT B, a cytoplasmic enzyme (Kleff et al., 1995; Brownell and Allis, 1996). HAT A is responsible for transcriptionally active chromatin-associated acetylation. Nuclear HAT A is bound to chromatin and acetylates all core histones when free or within nucleosomes (Brownell and Allis, 1996). Dr. Allis and colleagues were the first to purify and clone a HAT A. There studies showed that *Tetrahymena* HAT A (p55) is homologous to yeast Gcn5, a transcriptional adaptor, that has HAT activity (Brownell et al., 1996). This important breakthrough provided a direct link between the process of transcription activation and histone acetylation.

Tetrahymena HAT A (p55) and yeast Gcn5 are components of large multiprotein complexes, and the substrate specificity of the catalytic subunit is regulated by the proteins binding to it (Grant et al., 1997). Yeast Gcn5 and *Tetrahymena* p55 can acetylate free histone H3 but these HAT As are unable to acetylate histones in nucleosomes. Yeast Gcn5 is a component of two high molecular mass complexes (0.8 and 1.8 megadaltons) (Grant et al., 1997). These high molecular mass, multiprotein complexes acetylated histones in nucleosomes and free histones. Both HAT A complexes contain Ada2 and Ada3. Gcn5-Ada2-Ada3 is a putative adaptor complex that connects DNA-bound transcription factors (activators) to components of the PIC (Candau et al., 1997). The HAT domain of yeast Gcn5 has been localized. Gcn5 requires both the HAT domain of Gcn5 and interaction with Ada2 for transcriptional activation (Candau et al., 1997). Human homologues of Gcn5 and Ada2 have been identified (Candau et al., 1996).

Tetrahymena HAT A and yeast Gcn5 have a bromodomain that is lacking in yeast Hat1p. The bromodomain, which is thought to be a protein-protein interaction domain, is found in the C-termini of these proteins (Haynes et al., 1992). Several other recently identified HAT As have the bromodomain, including $TAF_{II}250$ (a 250 kDa protein that binds to TATA-binding protein), CBP/p300 but not P/CAF (Yang et al., 1996; Bannister and Kouzarides, 1996; Mizzen et al., 1997; Ogryzko et al., 1997). It is possible that HAT As interact with other transcription factors through the bromodomain, directing HAT A to specific regions in chromatin and in the nucleus (Brownell and Allis, 1996).

Most HAT As are coactivators (e.g., Gcn5, $TAF_{II}250$, CBP/p300). CBP/p300 binds to hormone receptors, AP-1, c-*Myb*, SV40 large T antigen, and adenovirus E1a, and appears to be an integrator of multiple signalling pathways (Kamei et al., 1996; Avantaggiati et al., 1996; Oelgeschläger et al., 1996; Hanstein et al., 1996) (**Fig. 9**). Unlike Gcn5, *Tetrahymena* p55, $TAF_{II}250$, or P/CAF, CBP acetylates all four core histones in nucleosomes (Ogryzko et al., 1997). The discovery that several transcription modulators or coactivators have HAT activity provides a mechanism by which chromatin structure is altered in the vicinity of DNA-bound transcription activators. A variety of transcription factors including hormone receptors, CREB, and fos-jun will bind directly or indirectly to CBP, recruiting a coactivator with histone acetyltransferase activity (**Fig. 10**). The HAT activity of CBP would then acetylate surrounding histones in nucleosomes, leading to the destabilization of nucleo-

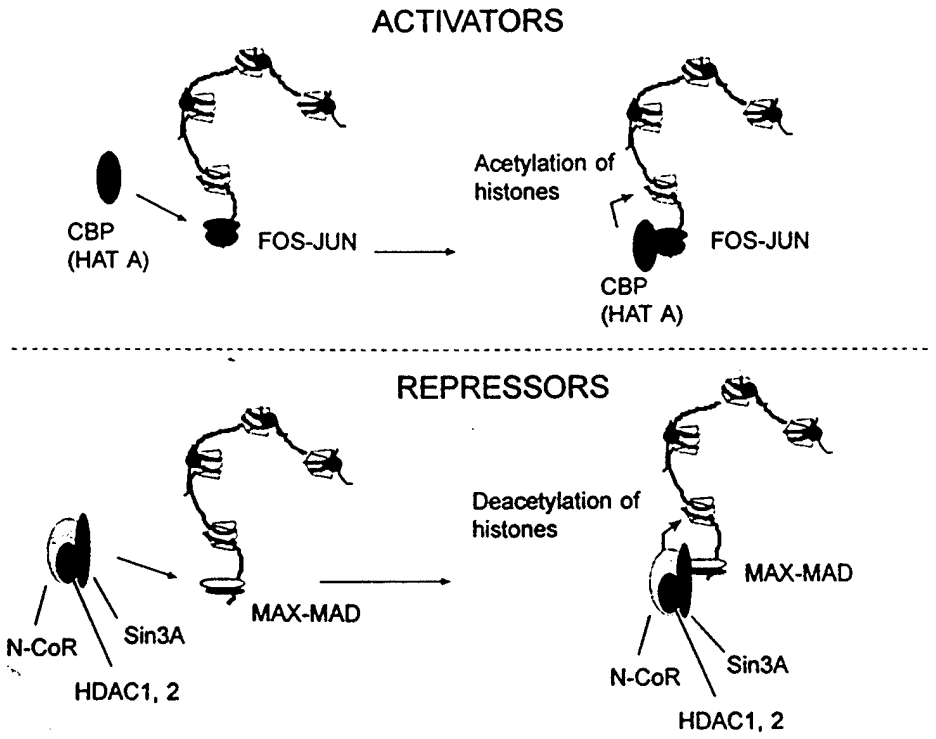

Figure 10. Role of HAT As and HDACs in Transcriptional Activation and Repression

Top panel: Fos-Jun is shown recruiting the coactivator CBP, resulting in the acetylation of nucleosomal histones. Bottom panel: Mad-Max is shown recruiting the corepressor HDAC multiprotein complex, resulting in the deacetylation of nucleosomal histones.

some and higher order chromatin structure. Such a chromatin state is thought to be facilitate the binding of other transcription factors and, in general, aid the transcription process.

XI. Histone deacetylase and gene repression

Histone deacetylases are nuclear enzymes that have been isolated from a variety of sources. Our studies have focused on the chicken erythrocyte histone deacetylase, an enzyme associated with the nuclear matrix (Hendzel et al., 1991). Chicken erythrocyte histone deacetylase is a component of a multiprotein complex that has a molecular mass in excess of 400 kDa (Li et al., 1996). The chicken histone deacetylase complex extracted from nuclei dissociates to a 66-kDa form in 1.6 M NaCl or when applied to an ion-exchange column (e.g., Q-sepharose). However, the high molecular mass histone deacetylase complex extracted from chicken erythrocyte nuclear matrices does not dissociate in 1.6 M NaCl, but this HDAC complex did dissociate to a 66-kDa form when applied to a Q-sepharose column (Li et al., 1996). These observations suggest that the solubilized nuclear matrix histone deacetylase is associated with proteins that stabilize the complex from dissociation into the 66-kDa form in a high concentration of salt.

The high molecular mass chicken erythrocyte histone deacetylase complex deacetylates the four core histones in chromatin, but has a preference for H2B (Li et al., 1996). Dissociation of the multiprotein histone deacetylase complex resulted in a change in substrate preference. The 66-kDa enzyme could not deacetylate histones in chromatin and had a preference for free H3. The data suggest that proteins important in regulating HDAC activity were lost during enzyme purification (Li et al., 1996).

Dr. Schreiber and colleagues were the first to clone a mammalian histone deacetylase (HDAC1, 55 kDa) (Tauton et al., 1996). They found that mammalian histone deacetylase was related to the yeast transcription regulator Rpd3p, providing a link between transcription regulation and histone deacetylation. At around the same time, Dr. Grunstein and colleagues purified two yeast histone deacetylase complexes, HDA (350 kDa) and HDB (600 kDa) (Carmen et al., 1996; Rundlett et al., 1996). The HDA complex consists of multiple peptides with molecular masses of approximately 70 kDa. Two peptides from the HDA complex have been sequenced, and yeast genes HDA1 (codes for p75) and HDA3 (codes for p71) isolated. HDA1 shares sequence similarity with Rpd3p, a yeast histone deacetylase. Gene disruptions of HDA1 or HDA3 resulted in the loss of the HDA, but not, HDB complex. Rpd3p is a component of HDB (Rundlett et al.,

1996). Rpd3p binds to Sin3 which in turn associates with Ume6, a DNA-binding protein required for the repression of several genes including those involved in meiosis (Kadosh and Struhl, 1997). These observations suggest that the yeast HDB complex consists of Rpd3p, Sin3 and Ume6.

Mammalian histone deacetylase HDAC1 is related to yeast transcriptional regulator Rpd3p (Tauton et al., 1996). Although HDAC1 has a reported molecular mass of 55 kDa, we have found that it migrates on our SDS polyacrylamide gels with an apparent molecular mass of 66 kDa. The mammalian homologue of Rpd3p, named HDAC2, has been cloned (Yang et al., 1996). Chicken erythrocyte histone deacetylase has been purified, and the enzyme migrates as a 66-kDa band on SDS gels (J.-M. Sun, H. Y. Chen, J. R. Davie, unpublished observations). Thus, chicken erythrocyte histone deacetylase has a molecular mass similar to that of mammalian HDAC1.

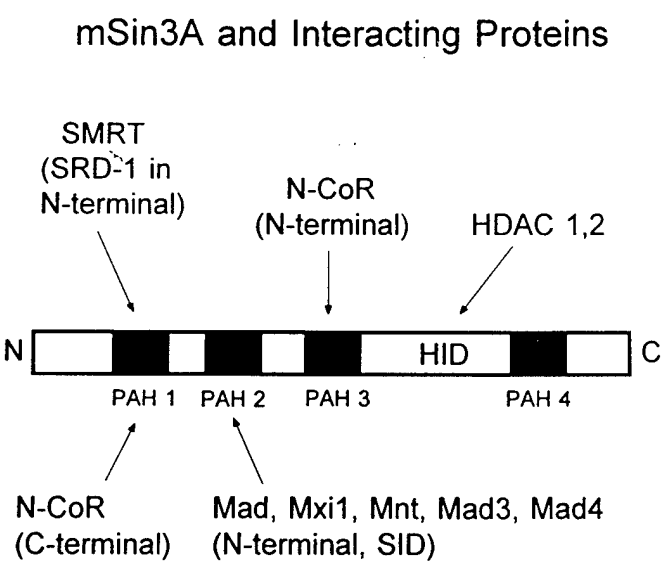

Figure 11. Regions of mSin3A Involved in Protein Interactions

N-CoR, SMRT and Mad family members interact with different paired amphipathic helix (PAH) domains in mSin3A. The HID domain which binds to HDAC 1 and 2 is shown.

As with yeast and chicken histone deacetylases, mammalian histone deacetylases, HDAC1 and HDAC2, exist as high molecular mass, multiprotein complexes (Hassig et al., 1997). HDAC1 and HDAC2 bind to a variety of proteins, including RbAp48, YY1, and mammalian (m) Sin3A and mSin3B (Tauton et al., 1996; Yang et al., 1996; Laherty et al., 1997). These HDAC-binding proteins may exist in different HDAC multiprotein complexes. For example, HDAC complexes with mSin3 do not contain YY1 (Zhang et al., 1997). HDAC1 was purified as a complex with RbAp48, a 50 kDa Rb-binding protein that binds to the C-terminus of unphosphorylated or hypophosphorylated Rb (Tauton et al., 1996; Qian et al., 1993). RbAp48 has several partners in addition to HDAC1. RbAp48 is a component of human and Drosophila CAF-1 (chromatin assembly factor 1) (Verreault et al., 1996). A yeast protein similar to RbAp48, Hat2p, is component of yeast HAT B (Roth and Allis, 1996; Parthun et al., 1996).

HDAC1 and/or HDAC2 are in large multiprotein complexes that contain mSin3, N-CoR, and SMRT, proteins that are corepressors (Nagy et al., 1997; Hassig et al., 1997; Laherty et al., 1997; Heinzel et al., 1997). Mammalian Sin3A and mSin3B have four paired amphipathic helix (PAH) domains thought to be involved in protein-protein interactions (**Fig. 11**). HDAC1 and HDAC2 bind to the region between PAH3 and PAH4, referred to as HID [the histone deacetylase interaction domain (HID)] (Laherty et al., 1997). The HID region is conserved in mSin3A, mSin3B and yeast Sin3. Mammalian Sin3A (150 kDa) interacts with many other proteins, including SAP18 (mSin3 associated protein), Mad family members (Mad1, Mad3, Mad4, Mxi1) and Max-binding repressor Mnt, SMRT, and N-CoR (Laherty et al., 1997; Zhang et al., 1997; Nagy et al., 1997; Heinzel et al., 1997; Alland et al., 1997). mSin3A does not have DNA binding ability; however, several of the proteins associated with mSin3A can direct it to specific DNA regulatory regions. The N-terminal region (SID, mSin3 interaction domain) of the Mad family members and Mnt binds to PAH2 of mSin3 (**Fig. 11**). Mad family members form a dimer with Max, a DNA-binding complex that binds to E-box related DNA sequences (Laherty et al., 1997). Max and Mad proteins are members of the basic region-helix-loop-helix-leucine zipper (bHLH-Zip) transcription factors. Myc forms a heterodimer with Max which binds to the same E-box-related DNA sequences as does Mad-Max heterodimers. However, Myc-Max activates genes, while Mad-Max represses their transcription. The repressive action of Mad-Max is mediated in part by the interaction of Mad with mSin3 which in turn is associated with HDAC1 and/or HDAC2. N-CoR and SMRT bind to unliganded retinoid and thyroid hormone receptors (Nagy et al., 1997; Heinzel et al., 1997). Thus, like the bHLH-Zip repressor proteins, unliganded hormone receptors recruit the HDAC multiprotein complex. HDAC has a principal role in transcription repression. Several studies show that tethering HDAC1 or 2 to a promoter by fusing HDAC to a DNA-binding domain (e.g., Gal4 DNA-binding domain) results in transcription inhibition (Yang et al., 1996; Zhang et al., 1997; Kadosh and Struhl, 1997; Nagy et al., 1997). These studies suggest that repressors recruit

histone deacetylase which would deacetylate histones in nucleosomes, leading to the condensation of chromatin (Wolffe, 1997) (**Fig. 10**).

Although these studies show that HDAC is involved in repression, HDAC is associated with transcriptionally active chromatin. Both HAT As and HDACs are needed to catalyze dynamic acetylation of histones associated with transcribed chromatin domains. The presence of both HAT As and HDACs at transcriptionally active regions allows the rapid manipulation of nucleosome and chromatin structure (Wade and Wolffe, 1997).

XII. Histone acetylation and nuclear matrix

Vertebrate histone acetyltransferase (HAT A) and histone deacetylase (HDAC) are associated with the nuclear matrix (Hendzel et al., 1991; 1992; 1994; Li et al., 1996). Nuclear skeletons from chicken immature erythrocytes retain 80% of the nuclear HAT A and HDAC activities, and these enzymes catalyze reversible acetylation using as substrate the chromatin fragments associated with the nuclear skeletons (Hendzel et al., 1994). These studies suggest that HAT A and HDAC are colocalized to specific sites on the nuclear matrix. However, there is no evidence that HAT A and HDAC are part of the same large complex. We proposed a model in which nuclear matrix-bound HAT A and HDAC mediate dynamic interactions between the nuclear matrix and transcriptionally active chromatin (**Fig. 8**) (Davie and Hendzel, 1994; Davie, 1995). We have evidence that HDAC1 is associated with the matrix, but the identity of the nuclear matrix bound HAT A is currently unknown. Several transcription factors binding directly or indirectly with HAT A and HDAC are nuclear matrix proteins. For example, YY1 is a nuclear matrix protein that binds to HDACs (Yang et al., 1996; Guo et al., 1995). Estrogen receptors bound to the nuclear matrix could recruit CBP, an HAT A (Hanstein et al., 1996). Determining how HATs and HDACs are recruited to nuclear matrix sites engaged in transcription will be an important challenge.

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Estrogen Receptor Diminishes DNA-Binding Activities of Chicken GATA-1 and CACCC-Binding Proteins

LAUREL T. HOLTH, JIAN-MIN SUN, AMANDA S. COUTTS, LEIGH C. MURPHY, and JAMES R. DAVIE

ABSTRACT

The estrogen receptor (ER) repressed erythroid differentiation and erythroid-specific gene expression. In this study, we investigated the effect of ERα (referred to throughout as ER) on DNA-binding activities of transcription factors involved in regulating the expression of erythroid-specific genes, and, in particular, the histone H5 gene. Using electrophoretic mobility shift assays, we found that in the presence of rabbit reticulocyte lysate, human ER reduced the binding activities of chicken immature erythrocyte nuclear extracted proteins to GATA and CACCC sites in the H5 promoter and enhancer. In contrast, the binding activities of NF1 and Sp1 were not affected by ER. Binding of ER to an estrogen response element was enhanced by addition of rabbit reticulocyte lysate. This lysate was also necessary for ER to diminish the DNA-binding activity of GATA-1. These results suggest that additional factor(s) are necessary for full ER function. Both GATA-1 and CACCC-binding proteins are critical for the developmentally regulated expression of erythroid-specific genes. We hypothesize that interference in DNA-binding activities of GATA-1 and CACCC-binding proteins is the mechanism by which the ER inhibits regulation of these genes.

INTRODUCTION

IN AN ESTROGEN-DEPENDENT MANNER, the estrogen receptor (ER) inhibits erythroid differentiation and expression of erythroid-specific genes in chickens (Schroeder *et al.*, 1993). Histone H5 is a H1-like histone. During differentiation and maturation of chicken erythrocytes, levels of H5 increase, and this is correlated with chromatin condensation, the shut down of replication and the repression of transcription of most genes (Sun *et al.*, 1989). The chicken H5 gene is expressed specifically in erythroid cells and is transcriptionally active in adult chicken immature erythrocytes (Affolter *et al.*, 1987). Partial repression of the H5 gene is observed in the presence of estradiol (Schroder *et al.*, 1993). We studied the effect of the ER on the DNA binding activities of proteins in chicken immature erythrocyte nuclear extracts that bind to regulatory sites of the H5 gene using electrophoretic mobility shift-binding assays (EMSA).

GATA-1 is a major regulator of erythroid genes, including the histone H5 gene (Pevny *et al.*, 1991). The effects of estrogens on erythropoiesis have been proposed to be a result of modulating GATA-1 activity through a protein–protein interaction with the ER (Blobel *et al.*, 1995). However, the interaction between ER and GATA-1 does not require ligand in *in vitro*-binding experiments, suggesting that binding is not ligand-dependent (Blobel *et al.*, 1995).

That GATA-1 is essential for erythroid-specific gene regulation has been well established: however, GATA-1 alone is not sufficient. The CACCC-binding protein binding site is also important for the regulation of genes involved in erythroid differentiation, including H5 and β-globin (Ren *et al.*, 1996). Using EMSA, we found that the ER interferes with the DNA-binding activities of erythroid proteins binding to the GATA and CACCC sites in the promoter and enhancer of the H5 gene. These effects were specific as ER did not affect binding of NF1 or Sp1. As both GATA-1 and CACCC-binding proteins are important for erythroid-specific gene expression, we postulate that ER interference in the binding of these transcription factors to the promoter and enhancer provides a mechanism for the inhibition of these genes.

MATERIALS AND METHODS

Preparation of nuclear extracts

Nuclear extracts from chicken immature erythrocytes were prepared as described previously (Sun *et al.*, 1992). Briefly,

Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba R3E 0W3, Canada.

adult White Leghorn chickens were made anaemic by injections of phenyl hydrazine hydrochloride. Nuclei were isolated by lysing erythrocytes in RSB buffer [10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 5 mM NaButyrate, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The nuclei were collected by centrifugation (1085 g, 10 min) and resuspended in RSB buffer with 0.25% (v/v) NP-40. Nuclei were transferred to a dounce homogenizer and homogenized five times. Nuclei were collected by centrifugation (1085 g, 10 min) and resuspended in RSB buffer with 0.25 M sucrose and then layered on top of RSB buffer containing 0.7 M sucrose. Nuclei were collected by centrifugation at 1935 g for 10 min. Nuclear extracts were made as described previously by extracting nuclei (200 A_{260} units/ml) with RSB containing 1 μM leupeptin, 0.15 μM aprotinin, and 0.3 M NaCl at 37°C for 10 min (Sun *et al.*, 1992). Extracts were centrifuged for 5 min at 12,100 g, and the supernatant was then dialysed against buffer D [20% (v/v) glycerol, 20 mM HEPES pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT] overnight. The dialysed supernatant was centrifuged 5 min at 12,100 g and then aliquoted and stored at −70°C.

In vitro transcription/translation of ER

The human ER was prepared by *in vitro* transcription/translation using the T_NT Coupled Rabbit Reticulocyte System (Promega). ER expression plasmid pT7βhER was a gift from Sophia Tsai (Baylor College of Medicine, Houston, TX). For a control, the *in vitro* transcription/translation reactions were repeated with T7 RNA polymerase that was heat killed before use by boiling for 5 min, producing a control lysate in which the ER was not transcribed. To control for the effect of the plasmid DNA *in vitro*, transcription/translation reactions were treated with DNase I (1 u/5μl of reaction) for 60 min at 37°C. The DNase I was inactivated prior to EMSA by adding 1 mM EDTA. Purified recombinant human ER produced in the baculovirus expression system was from PanVera Corporation (Madison, WI).

Electrophoretic mobility shift assays

The EMSA was done as previously described (Sun *et al.*, 1994) with minor modifications. Briefly, all EMSA reactions contained 10 mM Tris-HCl pH 7.5, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 4% (v/v) glycerol, 0.1 μg/μl bovine serum albumin (BSA), 0.05% (v/v) NP-40, 0.1 mM $ZnCl_2$, 0.05 μg/μl poly dIdC, and ^{32}P end labelled double-stranded oligonucleotide probe 10,000 chernokov counts per reaction. In addition, 7 μg or 10 μg chicken immature erythrocyte nuclear extract and 2 μl or 4 μl of *in vitro* transcribed/translated estrogen receptor or T7 heat killed *in vitro* transcribed/translated estrogen receptor were added where indicated. Purified human recombinant ER (PanVera Corp.) was also used in quantities indicated in some shifts. The same aliquot of rabbit reticulocyte lysate was used for both the reactions in which the ER was *in vitro* transcribed/translated and the control reaction. These reactions were always used in sets exposed to the same number of freeze–thaws. Variability in shifts was observed depending on the age of the various extracts and on the number of times they had been thawed and refrozen. Double-stranded oligonucleotides used in this study were the Sp1-oligonucleotide (high-affinity site in the H5 promoter, sequence 5′ GATGCCTGCGGGGCGGGGCAGAGGGGA 3′ and its complement), the NF1-oligonucleotide (NF1-binding site in the H5 E7 enhancer, sequence 5′ TCGAGGGCTTGGCACAGCCCCAAGACCAC 3′ and its complement), the GATA-oligonucleotide (GATA-1 binding site in H5 E7 enhancer, sequence 5′ GAGGCTGGAGATAACAGTGCGGC 3′, and its complement), and the ERE-consensus oligonucleotide (sequence 5′GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT3′ and its complement (Scott *et al.*, 1991). The oligonucleotides were end-labelled as described previously (Penner and Davie, 1992).

RESULTS

Effect of ER on activities of erythroid proteins binding to the GATA sequence

EMSA with nuclear extracts from chicken immature erythrocytes show four complexes with a labelled double-stranded oligonucleotide of the GATA-binding site of the E7 enhancer of the chicken H5 gene (Fig. 1). C1 consists of GATA-1 bound to the oligonucleotide, C2–C4 consist of GATA-1 complexed to other proteins with sizes estimated at 105 kDa, 26 kDa, 146 kDa, and greater than 450 kDa (Penner and Davie, 1992). The effect of ER on the presentation of the four complexes was studied by adding *in vitro* transcribed/translated ER to the nuclear extracts. Therefore, in addition to the ER, the reaction contained rabbit reticulocyte extract, T7 polymerase, and the ER expression vector DNA. An *in vitro* transcription/translation reaction using heat killed T7 polymerase (ER not synthesized) was used as a control.

In EMSA, we studied the effect of each component of the *in vitro* transcription/translation reaction on the formation of GATA-1 complexes. The rabbit reticulocyte extract increased the formation of complexes. The addition of BSA had a similar effect. Thus, we concluded that increased protein concentration enhanced protein–DNA interaction. In contrast, the ER expression plasmid DNA diminished complex formation. A search of the sequence for potential GATA-binding motifs identified several. We determined the effect of the ER expression plasmid DNA on the EMSA by treating the *in vitro* transcription/translation ER reaction with DNase I to degrade the DNA and then inactivated the DNase I with 1 mM EDTA prior to EMSA. When the ER expression plasmid DNA is degraded, the intensities of bands C1 and C3 is markedly increased (Fig. 1, Lanes 7 and 8). Thus, the ER expression plasmid is likely acting as a specific competitor. These studies show that components of the *in vitro* transcription/translation reaction have different effects on the formation of complexes. These observations underscore the importance of including the *in vitro* transcription/translation reaction with heat killed T7 as a control.

In the EMSA with the GATA oligonucleotide, the C2 and C4 bands disappeared when either *in vitro* transcribed/translated ER or the control was added indicating that a component of the *in vitro* transcription/translation reaction was responsible for preventing this GATA-1 multiprotein complex from forming. C3 was diminished by the addition of *in vitro* transcribed/translated ER (Fig. 1, Lanes 3 and 4) relative to the con-

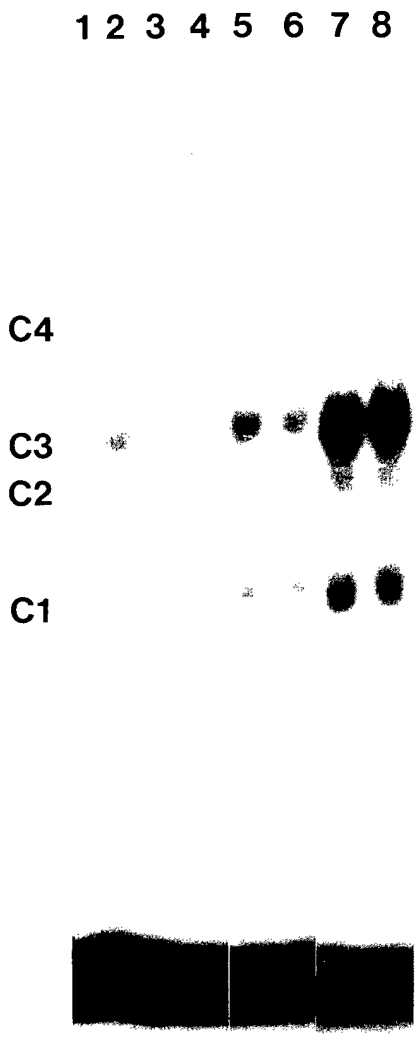

FIG. 1. EMSA with nuclear extract from chicken immature erythrocytes and GATA oligonucleotide. All lanes contain the labelled GATA oligonucleotide. Lanes 2–8 contain 7 μg of chicken immature erythrocyte nuclear extract. Lanes 3 and 4 additionally contain 2 and 4 μl, respectively, of the *in vitro* transcribed/translated ER. Lanes 5 and 6 contain 2 and 4 μl, respectively, of the control *in vitro* transcription/translation reaction which used heat killed T7 polymerase. Lane 7 and 8 contain 2 and 4 μl, respectively, of the *in vitro* transcribed/translated ER which has been treated with DNase I to degrade the ER expression plasmid DNA prior to adding to EMSA.

trol (Fig. 1, Lanes 5 and 6). This result suggests that the ER prevents the binding of GATA-1 to DNA. Less C3 was observed when greater amounts of either *in vitro* transcription/translation reaction were added although, to a lesser extent, for the control (Fig. 1, Lanes 3–6). This is attributed to the ER expression vector DNA containing GATA consensus sites that act as a cold competitor. The C1 band was slightly diminished when the *in vitro* transcribed/translated ER was added versus the control: however, the C1 band was not decreased further with increasing amounts of either. This may be because as more of the *in vitro* transcription/translation reaction was added, the increased protein concentration stabilized protein–protein interactions, resulting in an intensified band. This effect may have been competing with the decrease in C1 that would be expected with increased ER. Note that some variation was observed in the EMSA depending on the age and number of freeze–thaws to which the nuclear extracts and the *in vitro* transcription/translation reactions had been exposed. These results show that ER can interfere with GATA-1 DNA interactions.

ER does not affect activities of erythroid proteins binding to a NF-1-binding sequence

Next, we studied the effect of the ER on the DNA-binding activity of NF1 to a NF1 oligonucleotide. No differences between EMSAs containing the *in vitro* transcribed/translated ER versus the control were observed (Fig. 2, Lanes 3–6). Bands C1, C2, and C3 were not observed in EMSAs containing the *in vitro* transcription/translation mix, so no effect of ER on these bands could be determined. In contrast, band C4 was enhanced

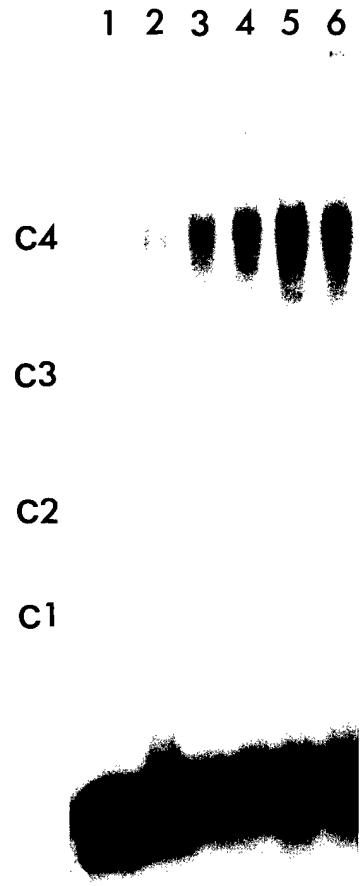

FIG. 2. EMSA with nuclear extract from chicken immature erythrocytes and NF1 oligonucleotide. All lanes contain the labelled NF1 oligonucleotide. Lanes 2–6 contain 10 μg of chicken immature erythrocyte nuclear extract. Lanes 3 and 4 additionally contain 2 or 4 μl, respectively, of the *in vitro* transcribed/translated ER. Lanes 5 and 6 contain 2 and 4 μl, respectively, of the control *in vitro* transcription/translation reaction which used heat killed T7 polymerase.

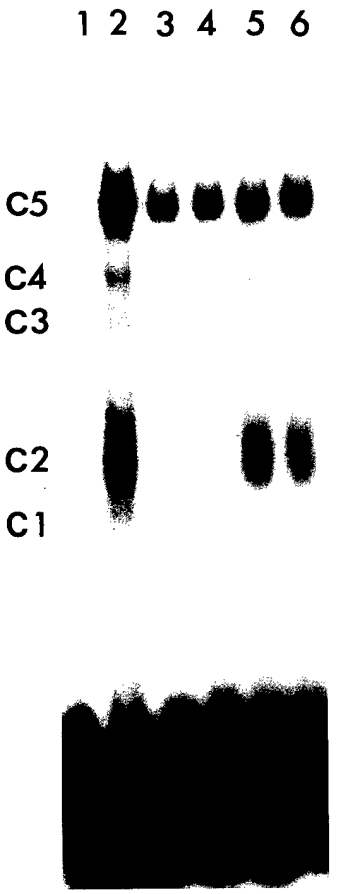

FIG. 3. EMSA with nuclear extract from chicken immature erythrocytes and Sp1 oligonucleotide. All lanes contain the labelled Sp1 oligonucleotide. Lanes 2–6 contain 10 μg of chicken immature erythrocyte nuclear extract. Lanes 3 and 4 additionally contain 2 or 4 μl, respectively, of the *in vitro* transcribed/translated ER. Lanes 5 and 6 contain 2 and 4 μl, respectively, of the control *in vitro* transcription/translation reaction which used heat killed T7 polymerase.

by the addition of either of the *in vitro* transcription/translation reactions.

Effect of ER on activities of erythroid proteins binding to the Sp1 and CACCC sequences

Previous work in our lab using anti-Sp1 antibodies and competitor oligonucleotides (Sun *et al.*, 1996) showed that an oligonucleotide with the sequence of the Sp1 high-affinity site of the H5 promoter, formed separate complexes with Sp1 and CACCC-binding protein. To determine if binding to Sp1 and CACCC sites was affected by ER, EMSA was done with a labelled Sp1 oligonucleotide (Fig. 3). Band C5 is due to Sp1 binding and band C2 is due to binding by the CACCC-binding protein (Sun *et al.*, 1996). In EMSA done with *in vitro* transcribed/translated ER versus with the control, band C5 was not significantly affected. In contrast, band C2 was eliminated by the addition of *in vitro* transcribed/translated ER (Fig. 3, Lanes 3 and 4), whereas control had no effect (Fig. 3, Lanes 5 and 6). Bands C1, C3, and C4 were not affected. These results indicate that ER can interfere with CACCC-binding proteins but not with Sp1 binding.

Effect of the control heat killed T7 in vitro transcription/translation reaction mixture on purified ER binding

To determine the effect of purified ER on GATA-1 binding to DNA, EMSA was done with purified human recombinant ER produced in the bacolovirus system. No effect was observed on the EMSA pattern of chicken immature erythrocyte nuclear extracts using a labelled GATA oligonucleotide when purified ER was added (Fig. 4). Addition of various concentrations of 17-β-estradiol from 10^{-9} M to 10^{-4} M also had no effect (data not shown). We, therefore, characterized the activity on the purified ER in EMSA using an ERE oligonucleotide. The purified ER (10 ng) did produce a shift with the ERE (Fig. 5, Lane 4): however, the intensity of this shift was greatly enhanced by adding the control *in vitro* transcription/translation reaction (Fig. 5, Lane 5). Addition of an equal amount of BSA also in-

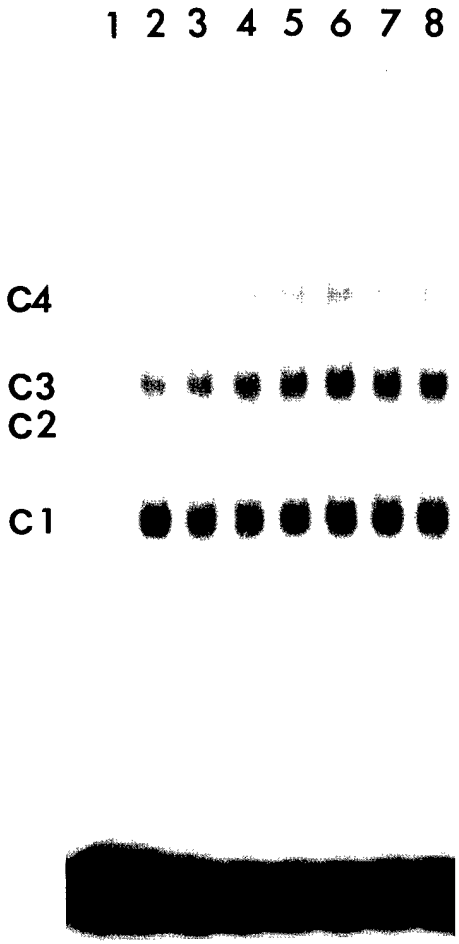

FIG. 4. EMSA with chicken immature erythrocyte nuclear extract and the GATA oligonucleotide plus increasing concentrations of purified human recombinant ER. All lanes contain the labelled GATA oligonucleotide. Lane 1 is probe alone. Lanes 2–8 contain 8 μg of nuclear extract. Lanes 3–8 contain 0.1, 1, 5, 10, 25, and 50 ng of purified ER, respectively.

FIG. 5. EMSA with labeled ERE oligonucleotide. All lanes contain the ERE oligonucleotide. Lane 1 probe alone. Lane 2, 4 μl of the control *in vitro* transcription/translation reaction which used heat killed T7 polymerase was added. Lane 3, 4 μl of the *in vitro* transcribed/translated ER was added. Lane 4, 10 ng of recombinant human ER was added. Lane 5, 10 ng of human recombinant ER and 4 μl of the control *in vitro* transcription/translation reaction which used heat killed T7 polymerase were added. Lane 6, 10 ng of human recombinant ER and 230 μg of BSA was added.

creased the intensity but not to the same extent (see Fig. 5, Lane 6). This result suggests that ER binding is not only affected by protein concentration but that a component of the *in vitro* transcription/translation reaction containing rabbit reticulocyte extract also contributes to ER-binding activity.

To determine if a component of the *in vitro* transcription/translation reactions was affecting the ability of the ER to interfere with erythroid proteins binding to the GATA sequence, EMSA with the GATA-1 oligonucleotide and chicken immature erythrocyte nuclear extracts was done with either the *in vitro* transcripted/translated ER, the control reaction with heat killed T7, or the control reaction plus the purified ER. When the control *in vitro* transcription/translation reaction was added to shifts with the purified ER, the effect was the same as for shifts done using the *in vitro* transcribed/translated ER (Fig. 6, Lanes 1–3). This activity was not due to an increase in protein concentration because when an equal concentration of BSA was added in place of the *in vitro* transcription/translation control reaction, no effect was observed. These results suggest that cofactors are important for the interaction of ER with GATA-1.

DISCUSSION

The ER in the presence of ligand inhibits expression of erythroid genes and differentiation of chicken erythrocytes (Schroeder *et al.*, 1993). Normal erythroid progenitors express ER (Schroeder *et al.*, 1993). In NIH 3T3 cells transiently transfected with ER, the transcriptional activity of murine GATA-1 is strongly repressed in the presence of estrogen (Blobel *et al.*, 1995). However, human ER binding to GST-GATA-1 did not appear to be ligand-dependent in *in vitro* assays (Blobel *et al.*, 1995). Blobel *et al.* (1995) suggested that the research for the difference in ligand dependence was that other proteins, absent in the *in vitro* assays, such as heat shock proteins, may affect the ability of ER to interact with GATA-1.

Blobel *et al.* (1995) were not able to detect murine GATA-1 interaction with the human ER in EMSA using nuclear extracts from COS cells transiently transfected with plasmids expressing GATA-1 alone or in combination with plasmids expressing human ER. We report here that human ER inhibits

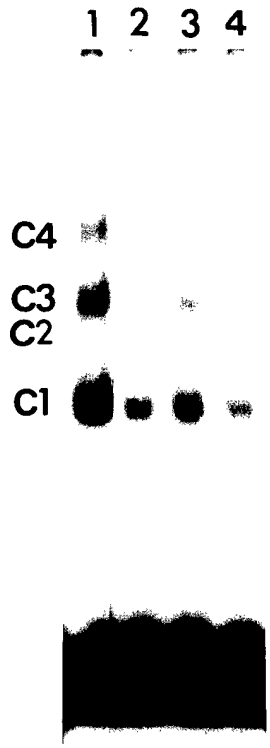

FIG. 6. EMSA with GATA oligonucleotide and nuclear extract from chicken immature erythrocytes, *in vitro* transcribed/translated ER versus the control *in vitro* transcription/translation reaction with heat killed T7 polymerase and recombinant ER. All lanes contain the GATA oligonucleotide and 8 μg of nuclear extract from chicken immature erythrocytes. Lane 2 additionally contains 2 μl of the *in vitro* transcribed/translated ER. Lane 3 additionally contains 2 μl of the control *in vitro* transcription/translation reaction which used heat killed T7 polymerase. Lane 4 additionally contains 2 μl of the control *in vitro* transcription/translation reaction which used heat killed T7 polymerase plus 10 ng of human recombinant ER.

the DNA-binding activities of GATA-1 multiprotein complex to GATA-binding site *in vitro*. This supports the hypothesis of Blobel *et al.* (1995) that binding of GATA-1 by ER prevents GATA-1 from binding to DNA. Possible reasons that we were able to observe an inhibition of binding to a GATA-1 site by ER using EMSA, while Blobel *et al.* (1995) did not, are as follows. First, unlike the ER which is highly conserved between species, murine and chicken GATA-1 are not very well conserved outside of the DNA-binding domain region, and interaction with the ER is species dependent. Second, Blobel *et al.* (1995) used nuclear extracts from COS cells cotransfected with GATA-1 and ER, whereas we used nuclear extracts prepared from chicken immature erythrocytes that naturally contain GATA-1 and GATA-1 multiprotein complexes to which was added exogenous ER synthesized *in vitro* in rabbit reticulocyte lysates. However, we were unable to see the ER effect on GATA-1 binding when purified recombinant ER was used. These results suggest that a factor necessary for the GATA-1 interaction with ER was absent in both the EMSAs of Blobel *et al.* (1995) and ours which did not contain the rabbit reticulocyte lysate.

In mouse erythroleukemia (MEL) cells transfected with the human α-globin and β-globin genes, expression of α-globin is constitutive, whereas β-globin expression is inducible. Addition of CACCC and GATA-1 motifs to the α-globin promoter (the only functional globin gene lacking these motifs) caused the α-globin gene to become inducible in MEL cells, however, separate addition of these sites did not (Ren *et al.*, 1996). It has been established that GATA-1 is essential but not sufficient for erythroid gene regulation (Ren *et al.*, 1996). GATA-1 and CACCC DNA-binding sites are both necessary for inducible regulation of globin genes in erythroid cells. This is particularly interesting in light of our finding that ER decreased the intensity of protein–DNA complexes formed in EMSA for both factors.

It has been reported that the ER can mediate gene activation in the absence of an ERE (Sukovich *et al.*, 1994). An example of this is the brain creatine kinase (BCK) promoter which is estrogen-responsive but contains no ERE. The ER is believed to form a protein–protein interaction with a factor that binds the BCK promoter (Sukovich *et al.*, 1994). Both GATA-1 and CACCC-binding protein are important in the regulation of the H5 gene and other erythroid genes. Here, we report that ER inhibits binding to their DNA-binding sites. We hypothesize that these interactions with the ER are responsible, at least in part, for the inhibitory action of estrogen on erythroid-specific genes and erythroid differentiation in chickens.

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Address reprint requests to:
Dr. J.R. Davie
Department of Biochemistry and Molecular Biology
Faculty of Medicine
University of Manitoba
Winnipeg, MB, Canada
R3E 0W3

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